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Genetic and Molecular Studies of *ced-5* and *ced-7*, Two Genes Required for Cell-Corpse Engulfment in *C. elegans*

by
Yi-Chun Wu
B.S., Chemistry
National Tsing Hua University

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Signature of author _____
Yi-Chun Wu

Certified by _____
H. Robert Horvitz
Thesis Supervisor

Accepted by _____
Frank Solomon
Chairman of the graduate committee
Department of Biology

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Abstract

One characteristic feature of programmed cell death is the engulfment of cell corpses. This process involves both the recognition and phagocytosis of cell corpses by engulfing cells. At least six genes are required for the engulfment of cell corpses in *C. elegans*. To understand the engulfment process, I have molecularly and functionally characterized two of these genes, *ced-5* and *ced-7*.

ced-5 encodes a protein similar to the human protein DOCK180 and the *Drosophila melanogaster* protein Myoblast City, both of which have been implicated in the extension of cell surfaces. The ectopic expression of a *ced-5* cDNA in the engulfing cells of a *ced-5* mutant rescued the engulfment defect, indicating that *ced-5* is likely to function in engulfing cells. *ced-5* mutants are defective not only in the engulfment of cell corpses but also in the migration of specific cells. Both engulfment and migration require a cell to extend its cell surface, consistent with the hypothesis that CED-5 protein functions in the extension of cell surfaces. The expression of human DOCK180 in *C. elegans* can rescue the cell migration defect seen in *ced-5* mutants, suggesting that *ced-5* and DOCK180 are functionally similar. CED-5 may function in the cytoskeletal reorganization that occurs as an engulfing cell extends its cell surface around a dying cell.

ced-7 encodes a protein similar to the family of ABC (ATP-Binding Cassette) transporters. The CED-7 protein is localized to the plasma membrane and is widely expressed during embryogenesis. Mosaic analysis of *ced-7* shows that *ced-7* functions in both dying cells and engulfing cells during the engulfment of cell corpses. CED-7 may function to translocate molecules mediating the interaction between cell surfaces of the dying and engulfing cells during engulfment.

Thesis supervisor: Dr. H. Robert Horvitz
Title: Professor of Biology

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Introduction

I. INTRODUCTION

Programmed cell death is a fundamental cellular process in the development of and homeostasis in both invertebrates and vertebrates (reviewed by Ellis et al., 1991c; Steller, 1995; Jacobson et al., 1997b). Programmed cell death serves several functions, including the shaping of tissues and organs, the removal of deleterious cells and the regulation of cell numbers to balance cell proliferation (reviewed by Ellis et al., 1991c; Steller, 1995; Jacobson et al., 1997b). Although diverse signals can induce apoptosis in a wide variety of cell types, a number of evolutionarily conserved genes regulate a common cell death pathway that has been conserved from nematodes to humans (reviewed by Jacobson, 1997a; Vaux, 1997). The significance and mechanisms of programmed cell death have been, and continue to be, the subjects of intensive investigation and review (Ellis et al., 1991c; Cohen et al., 1992; Raff, 1992; Raff et al., 1993; Smith et al., 1995; Steller, 1995; Thompson, 1995; Burek and Oppenheim, 1996; Chinnaiyan and Dixit, 1996; Fraser and Evan, 1996a; Fraser et al., 1996b; Osborne, 1996; Oppenheim, 1997; Jacobson, 1997a; Jacobson et al., 1997b; Jehn and Osborne, 1997; McCall and Steller, 1997; Webb et al., 1997; Winoto, 1997; Wong and Choi, 1997; Wyllie, 1997)

Programmed cell death is frequently also referred to as apoptosis. This term was introduced by Kerr *et al.* (1972) to describe a form of naturally-occurring cell death which is morphologically distinct from pathological necrotic cell death. During apoptosis the cytoplasm condenses, nuclear chromatin aggregates and the cell corpse is swiftly engulfed by another cell without lysis of the corpse. The integrity of organelles is well-maintained. By contrast, during necrosis organelles, including mitochondria, lysosomes and nuclei, swell and disintegrate and the plasma membrane ruptures, releasing intracellular macromolecules to the extracellular space.

One prominent feature of programmed cell death is the rapid engulfment and degradation of cell corpses. This process, which is important for resolution of inflammation as well as tissue remodeling, eliminates dying cells before they release potentially harmful contents (reviewed by Savill et al., 1993; Hart et al., 1996; Savill, 1997). Failure in engulfment has been implicated as a cause of certain inflammatory diseases (Savill et al., 1993; Savill et al., 1997), while ectopic engulfment could be an underlying cause of some degenerative diseases (see text below; Kim, S., 1994).

But what signals define the dying cells? How do engulfing cells recognize that signal? How does that recognition result in engulfment? To address these

questions, I have been studying cell-corpse engulfment in the nematode *C. elegans*. I first describe below what is known about cell death in *C. elegans*. Next, I briefly review studies of cell-corpse engulfment in mammals.

II. CELL DEATH IN *C. elegans*

1. Advantage of the nematode *C. elegans* in studies of cell death

C. elegans is a free living worm that feeds on bacteria and can be easily maintained in the laboratory (Brenner, 1974). Several features make *C. elegans* an excellent organism for the study of programmed cell death. The animal is transparent; therefore all the cell divisions and deaths can be observed in living organisms using Nomarski optics. The complete and essentially invariant cell lineage has been determined (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). During the development of a hermaphrodite, 131 of the 1090 somatic cells generated undergo programmed cell death. The specific time and place of death is known for each cell genetically programmed to die, thus allowing the study of cell death with single cell resolution. Powerful molecular and genetic techniques such as germline transformation (Mello et al., 1992) and mosaic analysis (Hedgecock and Herman, 1995) have been developed. Combining these techniques with detailed knowledge of the developmental anatomy of *C. elegans* has allowed rapid progress in our understanding of different aspects of cell death.

2. Morphology and kinetics of programmed cell death in *C. elegans*

As cells undergo programmed cell death, they adopt refractile and raised button-like morphology that is readily distinguishable under Nomarski optics. Time course ultrastructural studies of dying cells indicate that programmed cell death in *C. elegans* is morphologically similar to apoptotic cell death in vertebrates (Robertson and Thomson, 1982). Similarities include condensation of cytoplasm, aggregation of nuclear chromatin and engulfment by other cells (Robertson and Thomson, 1982). The recognition and engulfment of dying cells is a highly efficient process: dying cells are engulfed and digested usually within an hour of their first increase in refractility under Nomarski optics. Ultrastructural studies also show that the engulfing cell can recognize the dying cell before it adopts any visible morphological change, and even before the cell division generating the dying cell has been completed (Robertson and Thomson, 1982). Therefore, the engulfment-inducing signal must be generated during an early stage of the death process. In *C. elegans*, no cells function primarily as phagocytes and this role is served by many different cell types, including muscle

cells, intestinal cells and epithelial cells. During embryonic cell death the engulfing cells are often the sister cells of the dying cells. By contrast, the epithelial cells of hypodermis are usually responsible for the engulfment of postembryonic cell corpses.

3. Genetic and molecular analysis of programmed cell death in *C. elegans*

(1) The genetic pathway for programmed cell death

Genetic analysis has led to the identifications of 15 genes that affect programmed cell death (Figure 1). These genes define a genetic pathway with four distinct steps (reviewed by Ellis et al., 1991c; Hengartner and Horvitz, 1994b; Horvitz et al., 1994). Mutations in 12 genes affect all 131 programmed cell deaths. These *ced* (*ced* for cell death abnormal) genes define three steps of programmed cell death: execution of death, engulfment of cell corpses by neighboring cells and degradation of cellular debris. The other two *ces* (*ces* for cell death specification) genes and *egl-1* (egg-laying defective) gain-of function mutations define the step which determines specific subsets of cells to die or live.

(2) Genes required for the execution of death

At least three genes, *ced-3, 4* and *9*, function in the execution of cell death. Loss-of-function (lf) mutations in the *ced-9* gene cause excessive cell deaths, whereas a gain-of-function (gf) mutation in the *ced-9* gene protects cells from programmed cell death (Hengartner et al., 1992). The *ced-9* gene encodes a protein with sequence similarity to the mammalian proto-oncogene *bcl-2* (Hengartner and Horvitz, 1994a). Overexpression of *bcl-2* can prevent programmed cell death in *C. elegans* and can substitute for *ced-9* in *ced-9* mutant animals, suggesting that *bcl-2* and *ced-9* are functionally similar. Recent studies have identified a number of proteins with sequence similarity to Bcl-2, constituting a family (reviewed by Dietrich, 1997; Hawkins and Vaux, 1994; Hockenbery, 1994; Jacobson, 1997a; Reed, 1997). The mammalian members of this family contain not only the CED-9-like death-inhibiting (anti-apoptotic) proteins, such as Bcl-2 (Cleary et al., 1986), Bcl-xL (Boise et al., 1993), Mcl-1 (Reynolds et al., 1994), Bag-1 (Takayama et al., 1995), Bcl-w (Gibson et al., 1996), A1 (Lin et al., 1996) and NR-13 (Mangeney et al., 1996), but also the death-promoting (pro-apoptotic) proteins, such as Bax (Oltvai et al., 1993), Bak (Chittenden et al., 1995), Bik (Boyd et al., 1995), Nbk (Han et al., 1996), Bad (Yang et al., 1995) and Bid (Wang et al., 1996). Furthermore, many members of this family can interact to form heterodimers, and it has been suggested that this

heterodimerization may modulate the killing or protecting effects of these proteins (Oltvai et al., 1993; Oltvai and Korsmeyer, 1994; Sato et al., 1994; Yin et al., 1994; Sedlak et al., 1995; Yang et al., 1995; Zha et al., 1996; Diaz et al., 1997; Zha et al., 1997).

Two genes, *ced-3* and *ced-4*, are required for the execution of cell death (Ellis and Horvitz, 1986). Mutations in either of these genes block the deaths of almost all cells and also suppress the phenotype of excessive cell deaths in *ced-9*(lf) mutants. These observations suggest that *ced-9* negatively regulates the activity of *ced-3* and *ced-4* (Hengartner et al., 1992). Furthermore, the ability of *ced-3* to kill in the absence of *ced-4* but not *vice versa* suggests that *ced-4*-mediated killing requires *ced-3* activity (Shaham and Horvitz, 1996). Since the *ced-3* and *ced-4* genes act cell-autonomously, as suggested by the genetic mosaic analysis (Yuan and Horvitz, 1990) and the ectopic expression studies (Shaham and Horvitz, 1996), these two genes appear to activate a self-destruction program in the cells destined to die.

The *ced-3* gene encodes a cysteine protease, a member of the caspase superfamily (Yuan et al., 1993; Xue et al., 1996) with substrate specificity similar to that of the human caspase CPP32 (Xue et al., 1996). Similar to other members of the caspase superfamily, CED-3 is synthesized as an inactive precursor and is converted to the active form through proteolytic processing (Xue et al., 1996). The regulation of and substrates for the caspase superfamily have since become subjects of intensive research in programmed cell death (reviewed by Dixit, 1996; Zhivotovsky et al., 1996; Kumar, 1997; Miller, 1997; Porter et al., 1997). For instance, the cleavage of DNA fragmentation factor (DFF) by CPP32 may trigger DNA fragmentation during apoptosis (Liu et al., 1997). The cleavage of the actin-binding protein gelsolin by CPP32 has been implicated in morphological changes during apoptosis (Kothakota et al., 1997).

The *ced-4* gene encodes a protein similar to the human Apaf-1 protein, but the CED-3 homologous region and the WD repeats of the Apaf-1 protein are not present in CED-4 (Yuan and Horvitz, 1992; Zou et al., 1997). Both CED-4 and Apaf-1 contain a putative P-loop nucleotide-binding site. This site seems to be functional in CED-4 because point mutations in this site abolish the proapoptotic activity of CED-4 in a number of experimental systems (Chinnaiyan et al., 1997; James et al., 1997; Seshagiri and Miller, 1997). Furthermore, the *in vitro* translated wild-type CED-4, but not the CED-4 point mutant in this site, appears to bind to

the ATP analog, FSBA (Chinnaiyan et al., 1997). This binding can be inhibited by excess ATP but not CTP, indicating the specificity of CED-4 for ATP.

How do CED-3 and CED-4 activate the death program? Biochemical experiments suggest that CED-3 and CED-4 physically interact *in vitro* and that processing of CED-3 into the active protease can be greatly enhanced in cells that also express CED-4 (Seshagiri and Miller, 1997; Wu et al., 1997). The incubation of partially purified CED-4 with CED-3 promotes CED-3 auto-processing (Chinnaiyan et al., 1997). This process may require the binding of ATP, since a P-loop mutation in CED-4 blocked CED-4-mediated auto-processing of CED-3 (Chinnaiyan et al., 1997). Although a complete set of CED-3 substrates has yet to be identified, the activation of CED-3 protease activity and subsequent cleavage of CED-3 substrates may lead to cell death.

How does the CED-9 protein intervene to negatively regulate the death program? CED-9 physically interacts with CED-4 *in vitro* (James et al., 1997; Spector et al., 1997; Wu et al., 1997), suggesting that CED-9 may inhibit the death program via its interaction with CED-4. It has been shown that CED-9 can be cleaved by CED-3 *in vitro* and the presence of at least one of the two cleavage sites is important for complete protection against cell death by CED-9 (Xue and Horvitz, 1997). One of the cleaved products of CED-9(68-280) still retains partial protection activity and is sufficient to allow interaction with CED-4. These results suggest that CED-9 may protect programmed cell death by two mechanisms. One is to inhibit CED-3 protease activity via an interaction involving its CED-3 cleavage site, and the other one is probably through a physical interaction with CED-4 (Xue and Horvitz, 1997). CED-3, CED-4 and CED-9 appear to colocalize in mammalian cells and yeast and can be coimmunoprecipitated from cellular extracts (Chinnaiyan et al., 1997; Wu et al., 1997). The subcellular localization of these three proteins in *C. elegans* is not yet defined.

Recent work by Shaham and Horvitz (1996) suggests that the *ced-4* gene produces not only a major transcript, *ced-4S*, which possesses killing activity as noted above, but also a minor transcript, *ced-4L*, which can prevent programmed cell death when overexpressed. The *ced-4S* and *ced-4L* differ in one splicing site, which generates extra 72 nucleotides in *ced-4L*. It remains to be determined how *ced-4L* fits into the *ced-9*-mediated protection mechanism, and what its relationship with *ced-3* and *ced-4S* may be.

Unlike *ced-3*(lf) and *ced-4*(lf) mutations, *ced-8*(lf) mutations delay programmed cell death and have little effect on prevention of cells from programmed cell death, suggesting that *ced-8* may act in a branch of the *ced-3* and *ced-4*-mediated death process or may be involved in the temporal control of programmed cell death (Hengartner, 1997; G. Stanfield, M. Hengartner, H.R.H., personal communication). The *ced-8* gene encodes a novel protein (G. Stanfield and H.R.H., personal communication).

(3) Genes required for the formation of cell corpses

The genes involved in this step may be effectors of the death machinery, some of which may well be substrates of CED-3 protease. So far, only one gene involved in this step, *ced-11*, has been identified (G. Stanfield and H.R.H., personal communication). Mutations in the *ced-11* gene alter the morphology of cell corpses in embryos. Unlike the refractile button-like cell corpses observed in wild-type animals, the cell corpses in *ced-11* mutants are non-refractile and look like vacuoles. Since cell death and engulfment still occur in *ced-11* mutants, the *ced-11* gene product is not required either to initiate programmed cell death or for the recognition of cell corpses by engulfing cells. The *ced-11* gene encodes a novel protein (G. Stanfield and H.R.H., personal communication).

(4) Genes required for the engulfment of cell corpses

Once cells undergo programmed cell death, their corpses are swiftly engulfed and degraded. At least six genes, *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7* and *ced-10* are involved in the engulfment process (Hedgecock et al., 1983; Ellis et al., 1991b). Mutations in any of these genes block the engulfment of many cell corpses and result in persistent cell corpses (Figure 2). Because the cells destined to die still die in the engulfment-defective mutants, the engulfment *per se* does not elicit cell death; instead, it serves to remove the cell corpses after cell death occurs. Genetic analysis suggests that the six engulfment genes fall into two groups: *ced-1, 6* and *7* in one group and *ced-2, 5* and *10* in the other (Ellis et al., 1991b). Single mutants or double mutants within the same group show weak engulfment defects; however, double mutants between the two groups show strong engulfment defects. One model consistent with these observations is that the two groups of genes are involved in two distinct but partially redundant pathways that lead to the recognition and phagocytosis of dying cells (Ellis et al., 1991b). For example, dying cells may express on their surfaces two different ligands recognized by two distinct receptors on engulfing cells. Only when both ligand-receptor groups are engaged is phagocytosis highly efficient and

reproducible. All of the engulfment genes except for *ced-1* show maternal rescue for the engulfment of embryonic cell death (Hedgecock et al., 1983; Ellis et al., 1991b), suggesting that these genes are expressed in both the germline and embryos.

Phagocytosis of dying cells includes the extension of pseudopodia from engulfing cells. Ultrastructural studies of cell corpses of *ced-1, 2, 5, 6, 7* and *10* mutants did not reveal pseudopodia enveloping unengulfed cells, suggesting that the six genes may be involved in the recognition or phagocytosis rather than just the degradation of cell corpses after engulfment (Hedgecock et al., 1983; Ellis et al., 1991b). Engulfment is similar to cell migration in that both processes require extension of cell surfaces in a polarized fashion. Interestingly, mutations in one group of engulfment genes, *ced-2, 5* and *10*, also affect the migration of a pair of gonadal cells, the distal tip cells (DTCs) (Hengartner, 1997; K. Nishiwaki, personal communication; Wu and Horvitz, 1997). The DTC-migration defect does not show maternal rescue in *ced-2, 5* and *10* mutants (Wu and Horvitz, unpublished results; Hengartner, 1997). The DTCs are located at the tips of the two gonadal arms and guide the extension of each growing gonadal arm during larval development (Kimble, 1981; Hedgecock et al., 1987). In *ced-2, 5* and *10* mutants, the DTCs frequently make extra turns or stop prematurely, resulting in abnormally shaped gonads. It is possible that *ced-2, 5* and *10* function in the engulfing cells and DTCs for the extension of cell surfaces required for cell-corpse engulfment and DTC migration, respectively.

The engulfment process can also be activated by ectopic programmed cell deaths caused by ectopic expression of *ced-3* or *ced-4* (Shaham and Horvitz, 1996). The necrotic deaths caused by dominant mutations in the degenerin gene, *mec-4*, can also induce the engulfment process, albeit in a highly inefficient way (Hengartner, 1997). In *mec-4* (d) mutants, six touch neurons degenerate (Driscoll and Chalfie, 1991; Driscoll and Chalfie, 1992; Hall et al. 1997). The cell bodies of these neurons swell and can increase 100-fold in size during the degeneration process, which is presumably caused by osmotic imbalance. The genes *ced-2, 5* and *10* appeared to be most important among the six genes involved in the engulfment of the degenerative cell corpses (Hengartner, 1997; S. Chung and M. Driscoll, personal communication).

The six engulfment genes do not represent all genes involved in engulfment. The *ced-12* gene recently characterized by T. Schedl and M. Hengartner (personal communication) resembles *ced-2, 5*, and *10* in that it is

important for not only cell-corpse engulfment but also DTC migration. An additional screen for engulfment-defective mutants performed by Z. Zhou (Z. Zhou and H.R.H., personal communication) has identified several engulfment mutants with associated lethality. These mutants could define new engulfment genes, since lethal engulfment-defective mutants were not isolated by the previous screen (Ellis et al., 1991b). However, further mapping and characterization of these mutations is necessary to determine if they indeed define new engulfment genes.

Among the engulfment genes identified so far, three of them, *ced-6* (Q. Liu and M. Hengartner, personal communication), *ced-5* and *ced-7*, have been cloned. In Chapters 2 and 3, I present the molecular and functional characterization of the genes *ced-5* and *ced-7*, respectively.

(5) Genes required for the degradation of cell corpses

The *nuc-1* (nuclease deficient) gene is required to degrade the DNA of dead cells (Sulston, 1976; Hedgecock et al., 1983). In *nuc-1* mutants, the DNA of dead cells persists, although cell death and engulfment still occur. Partially purified cellular extracts from wild-type animals contain an endonuclease activity undetectable in extracts from *nuc-1* mutants (Hevelone and Hartman, 1988). This nuclease, presumably regulated or encoded by the *nuc-1* gene, is independent of Ca^{++} and Mg^{++} and prefers low pH (pH=4.5) for its optimal activity. The *nuc-1* gene is also required to digest the DNA of the bacteria on which the animals feed, since persistent DNA can be observed in the intestinal lumen of *nuc-1* mutants (Sulston, 1976).

In engulfment-defective mutants Feulgen-reactive material stays in the unengulfed cell corpses of *ced-1* and *ced-2* mutants (Hedgecock et al., 1983), suggesting that DNA degradation does not proceed or is incomplete when the engulfment process is blocked. Therefore, the complete digestion of the DNA from dead cells may require the participation of engulfing cells.

To study the degradation of the DNA from dying cells, I have adapted the TUNEL (TdT-mediated dUTP nick end labeling) technique (Gavrieli et al., 1992) for use in worms. The results generated using the TUNEL technique are presented in the appendix of my thesis. TUNEL has been widely used to identify dying cells in other organisms, since it specifically labels DNA ends, which are more abundant in cell corpses as a consequence of DNA degradation (Wyllie, 1980; Pandey et al., 1994; Walker and Sikorska, 1994). By studying the TUNEL-staining patterns in *nuc-1* and engulfment-defective mutants, I found that the

nuc-1 gene is likely to act in dying cells to degrade DNA during programmed cell death. I also identified differences among engulfment mutants in their effect on TUNEL-staining patterns.

(6) Genes required for the specification of cell death:

Two genes, *ces-1* and *ces-2*, are involved in the decision of a pair of pharyngeal cells, the NSM sister cells, to die or to live (Ellis and Horvitz, 1991a). In wild-type animals, NSM sister cells die. In *ces-1*(gf) or *ces-2*(lf) animals these cells frequently survive. *ces-1*(lf) mutations do not affect cell death in NSM sisters but are able to suppress the *Ces-2*(lf) mutant phenotype, suggesting that *ces-1* activity may be negatively regulated by *ces-2*. The *ces-1* gene encodes a zinc-finger protein (M. Metzstein and H.R.H., personal communication). The *ces-2* gene encodes a basic region leucine-zipper (bZIP) transcription factor and is most similar to members of the PAR subfamily of bZIP proteins (Metzstein et al., 1996). These findings that both CES-1 and CES-2 are likely to be transcription factors suggest that programmed cell death, like other cell fates, may be regulated at the transcriptional level. The target genes regulated by CES-1 and CES-2 remains to be determined.

Mutations in the *egl-1* (egg-laying-defective) gene specifically affect a pair of HSN (hermaphrodite-specific neurons) neurons, which innervate the vulval muscles and drive egg laying in hermaphrodites (Sulston and Horvitz 1977; Trent et al., 1983; Desai et al., 1988; Desai and Horvitz, 1989). In males, the function of the HSN neurons is not needed and these cells undergo programmed cell death. In *egl-1* (gf) hermaphrodites, the HSN neurons undergo programmed cell death, resulting in a defect in egg laying. Other cell deaths appear unaffected by *egl-1* (gf) mutations. It is possible that these mutations cause sexual transformation of HSN cell fate so that hermaphrodite HSN neurons adopt a male fate, which may lead them to activate the cell death program.

4. Germline cell death

Like somatic cells, cells in the germline also undergo programmed cell death. The germline in *C. elegans* is syncytial: germline nuclei are partially enclosed by membranes and not completely cellularized. Programmed cell death in the germline of the adult hermaphrodite appears to occur in the region where nuclei are arrested in the pachytene stage of meiosis but not in the mitotic region (M. Hengartner, E. Hartwieg and H.R.H., personal communication; Hengartner, 1997). Occasionally, mature oocytes have been observed to undergo programmed cell death. Time course studies using the light microscope and

ultrastructural studies of the electron micrographs of dying germ cells showed that dying cells cellularize and pinch off the syncytium at a very early stage of the death process (M. Hengartner, E. Hartwieg and H.R.H., personal communication; Hengartner, 1997), presumably to sequester the cell-killing molecules and to facilitate the removal of cell corpses.

Germline and somatic cell deaths are molecularly and morphologically similar. The *ced* genes described above are also important for germline cell death, although the function of *ced-11* in germline cell death has not been investigated. Germline cell corpses are recognized and engulfed by gonadal sheath cells (M. Hengartner, E. Hartwieg and H.R.H., personal communication; Hengartner, 1997). Unlike that in embryonic cell death, the engulfment defect in germline cell death does not show any maternal rescue in *ced-2, 5, 6, 7* and *10* mutants (Hengartner, 1997; Wu and H.R.H., unpublished results).

The *nuc-1* gene is also required for degradation of the DNA of germline cell corpses: large DAPI-positive DNA masses accumulate in the sheath cells, which are absent in wild-type animals (Hengartner, 1997).

5. Programmed cell death by murder

The cell deaths described above are cell suicides. This implies the cells kill themselves by producing cytotoxic product(s). Suicides constitute the vast majority of cell deaths in *C. elegans*. However, a small number of programmed cell deaths in males appear to die by murder; their neighboring cells are involved in the killing process.

The first case involves a symmetric pair of cells located in the midline of the animal, B.alapaav and B.arapaav (Note on nomenclature: in *C. elegans*, daughter cells are assigned their parent's name followed by a letter indicating their relative positions: a (anterior), p (posterior), d (dorsal), v(ventral) , l (left), r (right)). One of these two cells is murdered by P12.pa, since laser ablation of P12.pa prevents its death (Sulston and White, 1980). Mutations in *ced-1* and *ced-2*, which block the engulfment of cell corpses, can also prevent the cell death (Hedgecock et al., 1983), suggesting that the cell death in this case may be induced through engulfment by P12.pa.

The second case is the linker cell, which guides the growth of the male gonad to the tail, where the digestive and reproductive systems connect (Kimble and Hirsh, 1979). Once its job is fulfilled in late larval stage, the linker cell is murdered by either E.lp or E.rp cells. When both the E.lp or E.rp cells were killed by laser beam, the linker cell survived (Sulston and White, 1980). In *ced-3*

or *ced-4* mutants the linker cells die normally, suggesting that these two genes are not required for linker cell death. The role of engulfment in the murder remains to be determined. For example, it is not known if the linker cell persists in engulfment-defective mutants.

The third case involves another pair of symmetric cells in the male tail; either Br.ald or Br.ard is killed. However, this case is less firmly established as a murder since laser-ablating both the F.ld and F.rd cells thought to act in killing did not always prevent the cell death (Sulston and White, 1980).

6. Deaths that involve inappropriate engulfment in *lin-24* and *lin-33* mutant animals

Semi-dominant mutations in two genes *lin-24* and *lin-33* (*lin* for lineage abnormal) can cause P1.p-P12.p (collectively designated Pn.p) cells, which lie in the ventral midline and generate hypodermal and vulval cells, to adopt abnormal morphologies and degenerate (Ferguson and Horvitz, 1985; Ferguson et al., 1987; Kim, S., 1994). The death induced by *lin-24*(sd) and *lin-33*(sd) mutations does not seem to occur via the normal programmed cell death pathway, for two reasons. First, mutations in *ced-3* and *ced-4* do not block these deaths (Ellis and Horvitz, 1986). Second, these deaths appear to require the activities of the engulfment genes *ced-2, ced-5* and *ced-10* but not *ced-1, ced-6 and ced-7*, since mutations in any of the three genes suppress abnormal deaths in *lin-24*(sd) and *lin-33*(sd) mutants (Kim, S., 1994). It is possible that *lin-24* and *lin-33* mutations cause the Pn.p cells be recognized and engulfed by their neighbors as dying cells, a hypothesis that should be answerable using ultrastructural studies. The *lin-24* gene was cloned and encodes a novel protein (Kim, S., 1994). Further work to reveal the molecular identity of the *lin-33* gene and to explore the toxic nature of the LIN-24(sd) and LIN-33(sd) proteins is essential to understand the mechanisms underlying the abnormal deaths in *lin-24*(sd) and *lin-33*(sd) mutants.

III. CELL-CORPSE ENGULFMENT IN MAMMALS

I have described above what is known about cell death in *C. elegans*. Since my work is focused on the engulfment process, I will briefly review below what is known about the engulfment of cell corpses in other organisms.

Unlike the nematode *C. elegans*, flies (Tepass et al., 1994) and mammals (reviewed by Savill et al., 1993; Savill, 1997) have professional phagocytes, the macrophages, which recognize and engulf apoptotic cells. However, other cell types in mammals have also been observed to participate; for example, epithelial

cells (Dini et al., 1995), glomerular mesangial cells (Savill et al., 1992b) and fibroblasts (Hall et al., 1994) have been found to engulf neighboring apoptotic cells. Since mammalian macrophages are also responsible for phagocytosis of foreign invaders, it is possible that macrophages use similar recognition systems for apoptotic cells and foreign particles. However, several studies have indicated that different recognition systems are used in the two cases. Macrophage mannose receptor and receptors for opsonins (Fc, C3b and iC3b), which are involved in the recognition of foreign particles, are not required for phagocytosis of apoptotic cells (Savill et al., 1989). In addition, some molecules that abrogate the binding of macrophages to apoptotic cells have been tested and found to have no effect on macrophage phagocytosis of foreign particles. Furthermore, unlike phagocytosis of foreign particles, phagocytosis of apoptotic cells does not release phlogistic eicosanoid thromboxane or pro-inflammatory cytokines, both of which are important for inflammation (Weiss, 1989; Stern et al., 1996; Hughes et al., 1997).

Cell culture studies have implicated a number of molecules in cell-corpse engulfment. Here I briefly describe the types of cells and approaches that were used in these studies. The most commonly used apoptotic cells were aged lymphocytes, irradiated thymocytes and dexamethasone-treated thymocytes, all of which could be recognized and engulfed *in vitro* by monocyte-derived (unactivated) macrophages or thioglycollate-ilicited (activated) peritoneal macrophages. The main approach taken by these *in vitro* studies was to identify inhibitors that block the engulfment of apoptotic cells, thus implicating the inhibitor-targeted molecules in the engulfment process. Preincubation of apoptotic cells or phagocytes with inhibitors followed by a phagocytosis assay was a means to test the localization of the inhibitor-targeted molecules on either the surfaces of apoptotic cells or phagocytes.

The molecules implicated in the recognition of apoptotic cells are described below and summarized in Table 1. However, it is important to note that any role for these molecules in engulfment *in vivo* has yet to be tested.

1. Molecules implicated in the recognition of cell corpses

(1) Lectin-like proteins on engulfing cells

Cell adhesion mediated by binding of cell surface carbohydrates on one cell to lectins on another cell is one of mechanism underlying cellular interaction, which can be inhibited specifically by simple sugars recognized by lectins (Sharon and Lis, 1989). Lectin-like molecules may also be involved in recognition

of apoptotic cells by macrophages. The phagocytosis of mouse apoptotic thymocytes by mouse activated peritoneal macrophages can be inhibited by N-acetylglucosamine, its dimer N,N'-diacetylchitobiose, and to a lesser extent, galactose (Duvall et al., 1985). The specificity is indicated by a reduced or absent effect of other sugars in inhibiting macrophage phagocytosis of apoptotic thymocytes. Preincubation of macrophages with the inhibitory sugars inhibited phagocytosis, suggesting that the sugars were blocking a putative sugar-binding protein such as a lectin-like receptor on macrophages.

The ability to recognize sugar moieties on apoptotic cells may not be limited to macrophages. Non-progessional phagocytes in two cases have been found to recognize distinct sugar moieties of apoptotic cells. Apoptotic rat liver cells are engulfed by neighboring liver cells or hepatic sinusoid endothelial cells. Exogenous mannose (Dini et al., 1992; Dini et al., 1995), antibodies against asialoglycoprotein receptors (ASGPR) or ASGPR ligands (N-acetylglycosamine, galactose, lacosylated BSA and asialofetum) inhibited engulfment if engulfing cells were preincubated with these reagents. Preincubation of the apoptotic cells did not inhibit engulfment.

The second case is phagocytosis of apoptotic neutrophils by fibroblasts, which can be inhibited by mannan (a mannose-rich glycoprotein) and fucoidin (a sulfated fucose polysaccharide) (Hall et al., 1994). Since the expression of a mannosyl-fucosyl receptor is not detected in fibroblasts or neutrophils, a novel lectin-like molecule distinct from mannosyl-fucosyl receptor may be involved in recognition of apoptotic neutrophils by fibroblasts.

These data suggest that apoptotic cells undergo specific changes in surface carbohydrates as a consequence of apoptosis and these changes may lead to recognition and phagocytosis by engulfing cells (Duvall et al., 1985). Since N-acetylglucosamine and galactose are usually masked by terminal sialic acid residues in the sidechains of glycoproteins, loss of sialic acid has been proposed as a mechanism to expose specific sugar residues in apoptotic cells and trigger phagocytosis (Duvall et al., 1985).

(2) Vitronectin/CD36/ Thrombospondin on engulfing cells

Human monocyte-derived macrophages appear not to use lectin-like molecules for recognition of aged human neutrophils, since N-acetylglucosamine or other sugars did not inhibit this recognition (Savill et al., 1989). Instead, an integrin-mediated mechanism may be involved. Vitronectin and fibronectin

inhibited recognition and phagocytosis when preincubated with the macrophages, but not when preincubated with the neutrophils (Savill et al., 1990). Furthermore, engulfment can be inhibited by antibodies to the vitronectin receptor or by the tetrapeptide RGDS, suggesting a role for the vitronectin receptor in engulfment (Savill et al., 1990). The vitronectin receptor, along with the mannosyl-fucosyl receptor, may also be involved in the recognition of apoptotic neutrophils by human fibroblasts in culture (Hall et al., 1994).

The CD36 glycoprotein also may act in the engulfment of apoptotic cells by monocyte-derived macrophages. Anti-CD36 antibodies inhibited recognition and phagocytosis if preincubated with the macrophages, but not if preincubated with the aged neutrophils (Savill et al., 1991; Savill et al., 1992a). In addition, transfection of CD36 to monkey COS-7 cells or human Bowes melanoma cells, which have a limited capacity to engulf apoptotic cells, conferred greatly enhanced capacity to engulf apoptotic neutrophils and lymphocytes (Ren et al., 1995). The *Drosophila* Croquemort protein, which shares 23% identity with the murine CD36, has been implicated in cell-corpse engulfment (Franc et al., 1996). Transfection of COS-7 cells with a Croquemort cDNA conferred the ability to bind and engulf apoptotic thymocytes. The molecular identity of the CD36 or Croquemort ligands has yet to be determined.

Do the vitronectin receptor and CD36 cooperate in the recognition process? Savill *et al.* (1991 and 1992) showed that the vitronectin receptor and CD36 protein bind to apoptotic cells using the macrophage-secreted thrombospondin protein as a "bridge". Preincubation of either macrophages or apoptotic cells with anti-thrombospondin antibodies inhibited the binding of aged neutrophils (Savill et al., 1991), while purified thrombospondin potentiated their binding (Savill et al., 1991). Furthermore, thrombospondin can bind to both macrophages and aged neutrophils. This binding can be inhibited by the tetrapeptide RGDS, antibodies against CD36, or antibodies against the vitronectin receptor, suggesting that thrombospondin might act as a molecular bridge--one end binds to CD36 and the vitronectin receptor on macrophages and the other end binds to an as yet unidentified moiety on the aged neutrophils (Savill et al., 1991). The vitronectin receptor/CD36/thrombospondin recognition system may also be employed by macrophage engulfment of apoptotic thymocytes (Akbar et al., 1994) and eosinophils (Stern et al., 1996).

The moiety on the aged neutrophils recognized by monocyte-derived macrophages may not be proteinaceous, as binding was not affected by treating

aged neutrophils with a broad spectrum of proteases or inhibitors of protein synthesis. Instead, some charge-sensitive structures may be involved as the recognition is inhibited by cationic sugars and basic amino acids and is sensitive to pH (Savill et al., 1989).

(3) Phosphatidylserine receptor and scavenger receptor on engulfing cells

Under normal conditions, phosphatidylserine (PS) is almost totally confined to the inner leaflet of the plasma membrane. However, when cells undergo programmed cell death, PS is exposed to the outer leaflet (Fadok et al., 1992a; Fadok et al., 1992b; Martin et al., 1995). The phenomenon of the PS externalization during programmed cell death seems to occur widely, regardless the cell type or death-inducing stimuli (Martin et al., 1995). The externalized PS may trigger the engulfment of aged lymphocytes by activated macrophages, since this engulfment can be inhibited by liposomes containing PS but not by liposomes containing other anionic aminophospholipids (Fadok et al., 1992a; Fadok et al., 1992b). What is the mechanism of PS externalization? It has been suggested that the process involves down-regulation of the ATP-dependent aminophospholipid translocase, which normally maintains PS on inner leaflet of the bilayer, and the activation of an unidentified lipid scramblase (Verhoven et al., 1995).

What is the nature of the macrophage receptor(s) that recognizes and binds externalized PS on the surface of apoptotic cells? Macrophage membrane proteins such as the mouse macrosialin protein (Ramprasad et al., 1995), the class B scavenger receptors (CD36 and SR-BI) (Rigotti et al. 1995) have been shown to bind PS *in vitro*. CD36, as noted above have been suggested to function in the engulfment of apoptotic cells.

(4) 61D3 antigens on macrophages

The phagocytosis of apoptotic thymocytes, neutrophils and lymphocytes by monocyte-derived macrophages and activated peritoneal macrophages can be inhibited by the monocyte-specific monoclonal antibody 61D3, if the antibodies were preincubated with engulfing cells but not if preincubated with the apoptotic cells (Flora and Gregory et al., 1994; Pradhan et al., 1997). These data suggest that 61D3 antigens, yet to be identified, are present on the surface of both classes of macrophages and may be a component of the recognition mechanism.

(5) ABC1 protein on macrophages

The mouse ABC1 protein, a member of the ABC (ATP-binding cassette) transporter superfamily, is expressed in macrophages and the ability of macrophages to engulf apoptotic thymocytes, but not yeast cells, was severely impaired when macrophages were loaded with anti-ABC1 antibodies (Luciani and Chimini, 1996). While the ABC1 protein is able to transport anions in *Xenopus* oocytes (Becq et al., 1997), its physiological substrate(s) have not yet been identified. The *C. elegans* engulfment gene *ced-7* encodes a protein similar to ABC1. I will describe *ced-7* in chapter 3.

2. Multiple systems for recognition of apoptotic cells by engulfing cells

Different inhibitors appear to have effects in different experimental systems, as described above and summarized in Table 1. The recognition molecules used by different phagocytes were first compared by Fadok et al. (1992). Using various inhibitors described above, they suggested that activated macrophages used a PS receptor but not a vitronectin receptor for recognition of apoptotic neutrophils or lymphocytes, whereas monocyte-derived macrophages used a vitronectin receptor but not a PS receptor. Therefore, the recognition system used by these macrophages may depend on their state of activation. However, monoclonal antibody 61D3 can block engulfment by both kinds of macrophages (Flora and Gregory, 1994), suggesting that these phagocytes may share some common molecules in their recognition systems.

3. Phagocytosis of cell corpses

How does the binding of macrophages to apoptotic cells trigger phagocytosis? What is the motile force that extends the pseudopod in engulfing macrophages? Although answers to these questions are still unclear, lessons learned from macrophage phagocytosis of opsonized particles may provide us some clues. Depending on the types of opsonins, complement or antibody, two distinct mechanisms of phagocytosis are employed: the complement-opsonized particles sink into macrophages, while IgG-coated particles are engulfed by pseudopods projecting from the macrophage cell surfaces (Kaplan, 1977). Although the molecular basis for the difference between these modes of phagocytosis is unknown, the engulfment of cell corpses in *C. elegans* (Robertson and Thomson, 1982) and at least some, if not all, in mammals (reviewed by Wyllie et al., 1980) appears to be similar to engulfment of IgG-coated targets. Upon the binding of Fcγ receptors to Fc ligands, a number of signaling events occur, which lead to activation of gene expression, release of cytokines and cytoskeletal rearrangement (reviewed by Daëron, 1997). I briefly describe below

what is known or proposed for the signaling events in Fcγ receptor-mediated cytoskeletal rearrangement.

The binding of the Fcγ receptors to the Fc domains of the IgG-opsonized particles triggers receptor aggregation, which in turn activates the tyrosine kinases of the Src and Syk families (reviewed by Daëron, 1997; Strzelecka et al., 1997). These kinases phosphorylate Fcγ receptors and subsequently associate via their SH2 domains with the Fcγ receptors. The association of kinases with phosphorylated receptors may recruit substrates of these kinases such as p85 to the sites of the receptors (reviewed by Daëron, 1997; Strzelecka et al., 1997). It has been suggested that the phosphorylation of p85 leads to the activation of PI 3-kinase (Prasad et al., 1993; Yanagi et al., 1994), which catalyzes phosphorylation at the D-3 position of the inositol ring of phosphatidylinositol (PI), PI 4-phosphate and PI 4,5 biphosphate (reviewed by Toker and Cantley, 1997). One of the products, PtdIns(3,4,5)P3, may promote the activation of small GTP-binding proteins such as Rac, which may in turn stimulate actin polymerization (Wennstrom et al., 1994; Toker and Cantley, 1997). Protein kinase C (PKC) has also been found to be activated by Fcγ receptor aggregation (Zheleznyak and Brown, 1992). PKC and one of its substrate MARCKS (myristylated alanine-rich C kinase substrate), which cross-links F-actin and has been implicated in regulation of actin structures associated with membranes (reviewed by Aderem, 1992; Hartwig et al., 1992; Myat et al., 1997), are associated with membranes underlying phagocytic cups (Allen and Aderem, 1995). Therefore, the aggregation of Fcγ receptors may create plasma-membrane-associated actin-nucleation sites through recruitment of proteins that modulate actin structure and result in local rearrangement of the cytoskeleton.

Dynamic assembly of the actin cytoskeleton is essential not only for phagocytosis but also for cell movement (reviewed by Bray and White, 1988; Condeelis, 1993; Stossel, 1993; Burridge and Chrzanowska-Wodnicka, 1996). Therefore, phagocytosis and cell movement may share common sets of actin-binding proteins, including molecules that fragment (gelsolin), bundle (fimbrin and a-actinin) or cross-link (filamin) F-actin filaments, or molecules that sequester actin monomers from actin filaments (profilin) (reviewed by Bray and White, 1988; Barkalow and Hartwig, 1995; Lodish et al., 1995).

It is not yet clear if actin polymerization by itself is sufficient to generate motile force for phagocytosis, or if other motor molecules are required. Myosin II is localized to the membranes underlying phagocytotic cups (Stendahl et al.,

1980; Valerius et al., 1981) and myosin I is associated with F-actin on forming phagosomes (Allen and Aderem, 1995), implying that myosin I or II may provide mechanical force for phagocytosis. However, evidence for their direct involvement requires further research.

It is yet unclear to what extent the mechanisms of cytoskeletal rearrangement described above for Fcγ-induced phagocytosis may be conserved in engulfment of apoptotic cells. As noted above, a number of molecules have been identified that may mediate the recognition process in vertebrate cells, and which may facilitate investigation of the signaling events leading to activation of the actin-based phagocytosis machinery.

Do molecules implicated in cell-corpse engulfment in mammals have counterparts in *C. elegans*? As mentioned above and in chapter 3, the *C. elegans* engulfment gene *ced-7* encodes a protein similar to the mouse ABC1, which has been implicated in cell-corpse engulfment. The *C. elegans* genome sequencing project identified a sequence which may potentially encode a vitronectin receptor alpha subunit; however, its corresponding mutant phenotype is not known. Combining the powerful genetic approach such as screens for engulfment-defective mutants and the reverse genetics technique with the knowledge learned from mammals, *C. elegans* may provide a good tool to delineate the pathway involved in cell-corpse engulfment.

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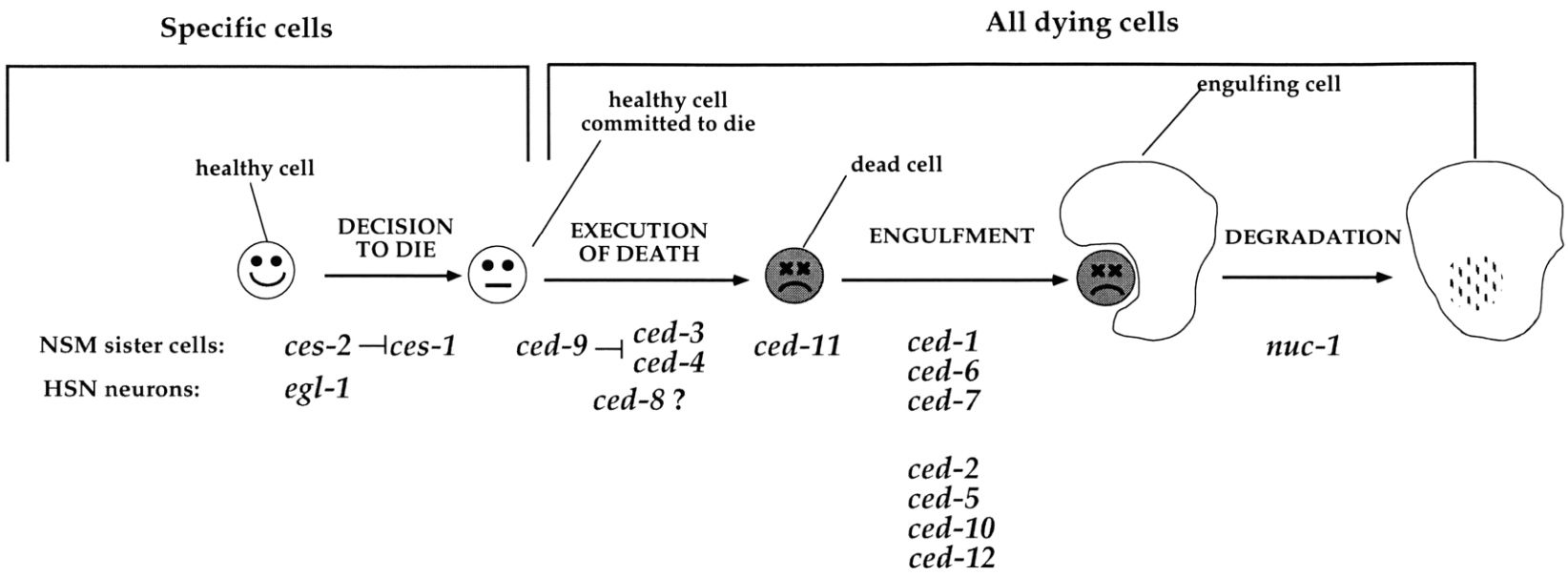
Figure 1 Programmed cell death pathway in *C. elegans*.

(→) positive regulatory interaction; (⊣) negative regulatory interaction.
See text for details. Adapted from Ellis et al. 1991c; Hengartner and Horvitz, 1994b; Horvitz et al., 1994.



CES-*1*: zinc-finger transcription factor
CES-2: bZip transcription factor

CED-11: Novel protein

CED-3: ICE-like protease
CED-4: Apaf-1-like protein
CED-8: Novel
CED-9: Bcl-2-like protein

CED-5: DOCK180-like protein
CED-7: ABC transporter

Figure 2 Nomarski photographs of a wild-type and a representative mutant defective in the engulfment of cell corpses.

(A) A wild-type embryo at the 4-fold stage showing no cell corpses.
(B) A *ced-7* embryo at the same stage showing many persistent cell corpses, some of which are indicated by arrows.

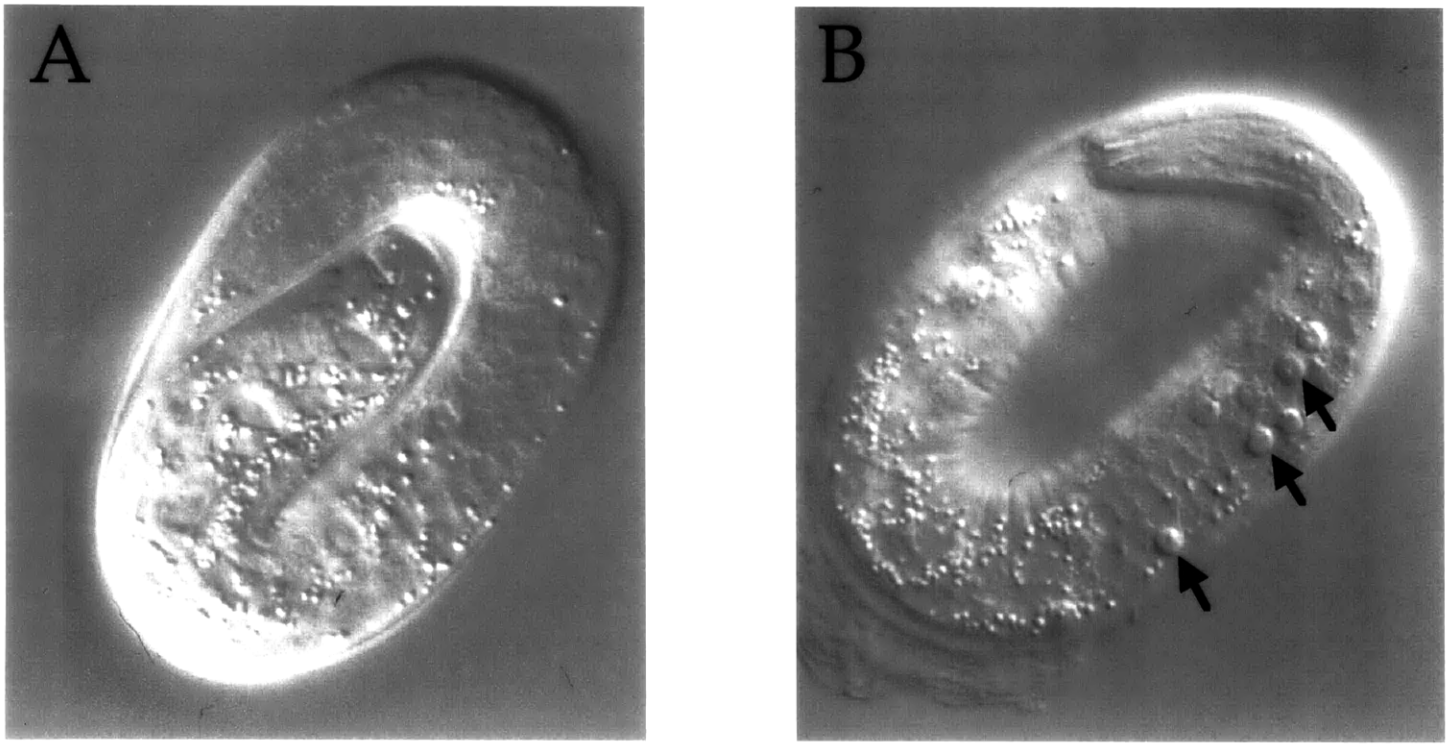

A
B

Table 1. Molecules implicated in the recognition of apoptotic cells in mammals

Mechanisms	species	Engulfing cells	Apoptotic cells	References
lectin-like molecule	mouse	activated peritoneal mø	irradiated thymocytes	Duvall et al., 1985
ASGPR/mannose receptor	rat	neighboring liver cell hepatic sinusoid endothelial	apoptotic liver cells	Dini et al., 1992 Dini et al., 1995
VNR/CD36/TMP	human mouse	monocyte-derived mø bone marrow mø mesangial cells	aged neutrophils dexamethason-treated dendritic cells thymocytes aged eosinophils	Savill et al., 1990 Savill et al., 1991 Savill et al., 1992a Akbar et al., 1994 Stern et al, 1996 Pradhan et al., 1997 Rubartelli et al., 1997 Hughes et al., 1997
VNR/mannan, fucoidin receptor	human	fibroblast	aged neutrophils	Hall et al. 1994
PS receptor (CD36 and SR-BI)	mouse	activated peritoneal mø	aged neutrophils irradiated thymocytes	Fadok et al., 1992a Fadok et al., 1992b
SR-A	mouse	thymic mø	dexamethasone-treated thymocytes	Platt and Gordon, 1995 Platt et al., 1996
mAb 61D3 antigen	human mouse	monocyte-derived mø activated peritoneal mø	aged neutrophils irradiated lymphocytes IL2-depleted T cells tonsillar germinal center B cells IL2-depleted T lymphocytes	Flora and Gregory, 1994 Pradhan et al. 1997
ABC1	mouse	activated peritoneal mø	irradiated thymocytes	Luciani and Chimini, 1996

Abbreviations: mø, macrophage; mAb, monoclonal antibody; PS, phosphatidylserine; SR-A, class A scavenger receptor; TMP, thrombospondin; VNR, vitronectin receptor.

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Chapter 2

C. elegans cell-corpse engulfment and cell-migration protein CED-5 is similar to human DOCK180 and *Drosophila* MBC

Yi-Chun Wu
H. Robert Horvitz

Howard Hughes Medical Institute
Department of Biology, 68-425
Massachusetts Institute of Technology
77 Massachusetts Avenue
Cambridge, MA 02139

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Abstract

During programmed cell death, cell corpses are rapidly engulfed[1-3]. The engulfment process involves the recognition and subsequent phagocytosis of cell corpses by engulfing cells. The molecular mechanisms responsible for the engulfment of cell corpses are largely unknown. We report that *ced-5*, a gene required for cell-corpse engulfment in the nematode *Caenorhabditis elegans*[4], encodes a protein similar to the human protein DOCK180 and the *Drosophila melanogaster* protein Myoblast City (MBC), both of which have been implicated in the extension of cell surfaces[5,6]. *ced-5* mutants are defective not only in the engulfment of cell corpses but also in the migration of two specific gonadal cells, the distal tip cells. The expression of human DOCK180 in *C. elegans* rescued the cell-migration defect of a *ced-5* mutant. We present evidence that *ced-5* functions in engulfing cells during the engulfment of cell corpses. Our findings lead us to suggest that *ced-5* acts in the extension of the surface of an engulfing cell around a dying cell during the engulfment process of programmed cell death. We name this new family of proteins that function in the extension of cell surfaces the CDM family, for CED-5, DOCK180 and MBC.

Introduction

Programmed cell death plays an important role in animal development and homeostasis[1,7,8]. The corpses of dead cells are swiftly eliminated from the body by the engulfment of neighboring cells[1-3]. In *C. elegans*, during the development of a hermaphrodite 131 of the 1090 somatic cells generated undergo programmed cell death[9-11]. Genetic analysis has led to the identification of at least six genes *ced-1, ced-2, ced-5, ced-6, ced-7* and *ced-10* (*ced*, cell death abnormal) that function in the engulfment of cell corpses[4,12]. Mutations in any of these genes block the engulfment of many cell corpses and hence cause a mutant phenotype characterized by persistent cell corpses. How does an engulfing cell recognize a dying cell among its viable neighbors? How does the recognition system trigger phagocytosis? What is the mechanism underlying phagocytosis? To answer these questions, we cloned and characterized the gene *ced-5*.

Results and Discussion

The *ced-5* gene is involved in both cell-corpse engulfment and DTC migration

During the course of characterizing *ced-5* mutants, we noticed that *ced-5* mutants are defective not only in the engulfment process of programmed cell death but also in a specific cell migration, that of the gonadal distal tip cells (DTCs) (Table 1). This defect has been independently observed by K. Nishiwaki and M. Hengartner (personal communications). The DTCs are located at the tips of the two gonadal arms and guide the extension of each growing gonadal arm during larval development[13,14]. We found that *ced-2* and *ced-10* but not *ced-1, ced-6* and *ced-7* mutants also have DTC-migration defect (Table 1): the DTCs frequently make extra turns or stop prematurely, resulting in abnormally shaped gonads. The DTC-migration defect is unlikely to result from the accumulation of persistent cell corpses in the soma or in the germline, since this defect was not observed in the engulfment-defective mutants *ced-1, ced-6*, or *ced-7* and was not suppressed by *ced-4* or *ced-9*(gf) mutations, which block programmed cell death (Table 1).

Molecular cloning of the *ced-5* gene

To understand how *ced-5* functions in both the engulfment of cell corpses and the migration of the DTCs, we cloned this gene. *ced-5* maps between *mec-3* and *him-8* on chromosome IV[4]. These two cloned genes define an approximately 100 kilobase (kb) region on the physical map[15,16] (P. Meneely, personal communication; Fig. 1). We used genomic DNA clones from this interval to rescue the Ced-5 mutant phenotype of persistent cell corpses in germline transformation experiments and localized the rescuing activity to a 10.6 kb genomic DNA fragment (Fig. 1). We isolated *ced-5* cDNAs using this fragment and defined the 5' end of the *ced-5* message using the RACE (rapid amplification of cDNA ends) method. The sequences of these cDNAs revealed a 1,781 amino acid open reading frame, a 5' end SL1 *trans*-spliced leader found at the 5' end of many *C. elegans* transcripts[17] and a 3' end poly(A) tract, indicating that we had identified a complete *ced-5* transcription unit. Genomic subclones lacking portions of this transcription unit failed to rescue the Ced-5 phenotype (Fig. 1). Northern blot analysis using a *ced-5* cDNA as a probe, showed a single band of 5.6 kb (data not shown), consistent with the size of the full-length *ced-5* cDNA. The expression of a *ced-5* cDNA in *ced-5(n1812)* animals under the control of *C. elegans* heat-shock promoters (*hsp*)[18] rescued the defects in both DTC migration (Table 3) and cell-corpse engulfment (Table 4; see below), indicating that the *ced-5* cDNA encodes a functional CED-5 protein. We identified molecular lesions in six *ced-5*

mutant alleles: three, *n1812*, *n2002*, and *n2691*, have nonsense mutations (at codon positions 28, 962, 1,145, respectively); one allele, *mu57*, has a single-base deletion (in codon 216); two other alleles, *n2098* and *n2099*, have single-base changes (in the splice-acceptor sequences of the sixth and eighth introns, respectively) (Fig. 2 and Table 2). The allele *ced-5(n1812)* is likely to be null, since this ochre mutation presumably eliminates more than 98% of the CED-5 protein and the engulfment defect of *n1812* homozygotes is indistinguishable from that of *n1812/sDf2* heterozygous animals[4].

The *ced-5* gene encodes a protein similar to human DOCK180 and *Drosophila* MBC proteins

The 1,781 amino acid sequence of the CED-5 protein is most similar to the sequence of the human protein DOCK180. These proteins share 26% identity over their entire lengths (Fig. 2). CED-5 and DOCK180 both share significant sequence similarities with the amino acid sequences predicted from the *Drosophila* gene *mbc*[6] (Fig. 2), the human cDNA clone KIAA0209 (ref. 19), the yeast open reading frame L9576.7 (accession number 664878) and a mouse EST sequence (accession number AA110899).

The human DOCK180 protein rescues the DTC-migration defect of *ced-5* mutants

To learn if human DOCK180 might be a functional homolog of CED-5, we tested the ability of *hsp*::DOCK180 to rescue the Ced-5 mutant phenotype. Induced expression of DOCK180 rescued the DTC-migration defect (Table 3) but did not rescue the corpse-engulfment defect (data not shown). Rescue of the abnormal DTC migrations in *ced-5* animals by DOCK180 suggests that CED-5 and DOCK180 may be functionally similar.

CED-5 may be functionally similar to DOCK180 and MBC

DOCK180 was isolated based upon its interaction with the cytoskeleton-associated adaptor protein CRK[5] , which consists mainly of SH2 and SH3 domains[20] and has been implicated in integrin-mediated signaling and cell movement[21]. The expression of DOCK180 in 3T3 fibroblasts can cause these cells to extend their surfaces and adopt flat and polygonal shapes, indicating that DOCK180 can regulate the extension of cell surfaces[5]. *Drosophila* MBC is necessary for myoblast fusion and some epithelial cell migrations, both of which

require the extension of cell surfaces, presumably through a re-organization of the cytoskeleton[6,22]. By analogy, we postulate that *ced-5* functions to mediate the extension of cell surfaces and does so by acting both in migrating DTCs and in engulfing cells during the phagocytosis of cell corpses.

The *ced-5* gene may function in engulfing cells during the engulfment of somatic cell corpses

If *ced-5* indeed acts in engulfing cells, the expression of a wild-type *ced-5* gene in engulfing cells might rescue the engulfment defect of a *ced-5* mutant. As noted above, we rescued the Ced-5 engulfment defect using heat shock to induce the expression of an *hsp*::*ced-5* transgene (Table 4). We believe that this rescue was effected by the expression of *ced-5* in engulfing cells rather than in cell corpses, for two reasons. First, animals subjected to heat-shock at various stages of larval development were rescued, so that cell corpses generated during embryogenesis would have persisted for hours (in L1 and L2 larval stages) or even days (in L3 and L4 larval stages) in some rescued animals. The finding that such late expression, at a time when cell corpses have presumably long been dead, still rescued (Table 4; larvae column) suggests that *ced-5* functioned not in cell corpses but rather in engulfing cells. Second, using the green fluorescent protein (GFP) as a reporter[23], we found that *ced-5(n1812)* animals carrying a GFP transgene under the control of these *C. elegans* heat-shock promoters did not express GFP in persistent cell corpses following heat shock but did express GFP in other somatic cells, including engulfing cells, throughout larval development (data not shown). This finding indicates that the transcriptional and/or translational machineries are likely inactive in persistent cell corpses. We therefore conclude that rescue of the *ced-5* engulfment defect by the *hsp*::*ced-5* transgene was likely caused by the expression of this transgene in engulfing cells.

The *ced-5* gene may function in engulfing cells during the engulfment of germline cell corpses

We used a similar strategy to ask if *ced-5* functions in engulfing cells during germline as opposed to somatic cell death. Germline cell corpses are engulfed by the gonadal sheath cells in a *ced-5*-dependent manner (M. Hengartner, E. Hartwieg and H.R.H., unpublished observations). Following the heat shock of *ced-5* animals carrying an *hsp*::GFP transgene, we detected GFP expression in gonadal sheath cells but not in the germline (data not shown), consistent with

previous observations that these *C. elegans* heat-shock promoters do not drive the expression of transgenes in the germline[24] (A. Fire, personal communication). *ced-5* animals carrying an *hsp*::*ced-5* transgene showed significantly reduced numbers of persistent germline cell corpses following heat shock as compared to heat-shocked *ced-5* animals carrying an *hsp*::GFP transgene (Table 4). This rescue of the engulfment defect by an *hsp*::*ced-5* transgene in germline cell death further suggests that *ced-5* functions in engulfing cells during programmed cell death.

The expression of the CED-5 protein is not dependent on the process of programmed cell death

To test if the expression of *ced-5* is regulated by the process of programmed cell death, we examined CED-5 protein levels in *ced-3* (ref. 25), *ced-4* (ref. 25) and *ced-9*(gf)[26] mutants, all of which lack programmed cell deaths. Using affinity-purified anti-CED-5 antibodies, we detected a protein of approximate molecular mass 185K, slightly smaller than the 204K size predicted from the CED-5 amino acid sequence, from a wild-type embryonic extract on a western blot (Fig. 3). This protein was missing from extracts of the *ced-5(n1812)* ochre mutant (Fig. 3), confirming that it is indeed the product of the *ced-5* gene. The CED-5 protein level in *ced-3*, *ced-4*, and *ced-9*(gf) mutants was unaltered compared to that in wild-type animals (Fig. 3), indicating that the expression of CED-5 was not dependent on the execution of programmed cell death.

CED-5, DOCK180 and MBC may define a new gene family

We have shown that *ced-5* is likely to act in engulfing cells during the engulfment of cell corpses and also is required for the normal migration of the DTCs. Both cell-corpse engulfment and cell migration require a cell to extend its cell surface in a polarized fashion (Fig. 4), consistent with the hypothesis that CED-5, like DOCK180 and MBC, functions in cell-surface extension. Based upon these observations and the finding that DOCK180 rescued the DTC-migration defect of *ced-5* mutant animals, we propose that CED-5, DOCK180 and MBC define a new evolutionarily conserved gene family involved in the extension of cell surfaces. We call this family CDM (CED-5, DOCK180 and MBC). We propose that *ced-5* acts in phagocytosis in response to the recognition of dying cells during programmed cell death. By analogy to MBC, *ced-5* could mediate the cytoskeletal reorganization that occurs as an engulfing cell extends its cell surface around a dying cell during phagocytosis. We suggest that like other proteins

involved in programmed cell death[7], CED-5 and the proteins with which CED-5 interacts during the process of cell-corpse engulfment are likely to be evolutionarily conserved.

Methods

Germline transformation experiments

For genomic rescue experiments, DNA was co-injected into *ced-5(n1812)* animals at concentrations of 25-50 μg ml^{-1} with the dominant roller marker pRF4 (50 μg ml^{-1}), as previously described[27]. To determine the extent of rescue, we scored persistent cell corpses in the head regions of L2 or L3 roller animals from stably transmitting transgenic lines using Nomarski optics, as previously described[27]. Non-rollers or non-rescued rollers had about 20-25 cell corpses. Roller animals containing 0-5 corpses were scored as rescued for the *ced-5* engulfment defect.

Plasmid construction

To construct *hsp*::*ced-5*, we did a three-component ligation reaction, as follows. We ligated the *Kpn* I-*Xho* I fragment from the plasmid pC5OKBA, which contains the 5' half of the *ced-5* cDNA, with the *Xho* I-*Apa* I fragment from the pC583 construct, which contains the 3' half of the *ced-5* cDNA, to the *Kpn* I-*Apa* I fragment from *hsp* vectors pPD49.78 or pPD49.83 (from A. Fire). To construct *hsp*::GFP, we excised the *Xba* I-*Apa* I fragment from the plasmids pPD96.04 (from A. Fire) and Tu#61 (from M. Chalfie); these plasmids contain the GFP gene with and without a nuclear localization signal, respectively. We cloned the fragments into the vectors pPD49.78 and pPD49.83 previously digested with *Nhe* I and *Apa* I. To make *hsp*::DOCK180 constructs, we excised the *Xho* I fragment from the plasmid pBlDOCK180, which contains a DOCK180 cDNA[5], blunt-ended the fragment and cloned it into the vectors pPD49.78 and pPD49.83 via their *Eco*R V sites. The heat-shock constructs were co-injected into *ced-5(n1812); unc-76(e911)* animals at concentrations of 50 μg ml^{-1} with the *unc-76*-rescuing plasmid p76-16B[28] to establish transgenic lines and the *egl-5*::GFP plasmid pSC212 (A. Chisholm and H.R.H., unpublished results) to identify transgenic embryos (50 μg ml^{-1} each).

Heat-shock experiments

To determine the extent of rescue of the *ced-5* engulfment defect in somatic cell death, we subjected mixed-staged transgenic animals to a 1.5 hr. heat-shock

treatment at 33°C, and after allowing a 10 hr. recovery at 20°C we scored the number of persistent cell corpses in the head regions of transgenic animals at different stages. To test for rescue of the engulfment defect in germline cell death, transgenic animals were subjected to two pulses of heat shock to span an approximately 36 hr. period of germline cell death before scoring. In brief, we picked L4 transgenic animals and subjected them to a 1.5-hr. heat-shock treatment at 33°C. Following heat shock, animals entering adulthood were picked from Petri dishes and transferred to a 20°C incubator for recovery. After 10.5 hours, we subjected these animals to another 1.5 hr. heat-shock treatment at 33°C. The number of germline cell corpses in each gonadal arm was scored 22.5 hours after the second heat-shock treatment. To test for rescue of the DTC-migration defect, L2 transgenic animals were picked and subjected to three 1.5 hr. heat-shock treatments at 33°C separated by two 10.5 hr. recovery intervals at 20°C to span an approximately 25 hr. period during DTC migration. The DTC-migration pattern was inferred from the gonadal morphology of young adults.

Characterization of *ced-5* genomic and cDNA structure

The 10.6 kb *Cla* I genomic fragment containing *ced-5* rescuing activity was used to screen a mixed-staged cDNA library[29] and an embryonic cDNA library[30]. We determined the sequences of *ced-5* genomic DNA and three overlapping *ced-5* cDNAs, using the Sequenase 2.0 kit (USB). We identified the 5' end of the *ced-5* mRNA using the 5' RACE system (GIBCO-BRL). We identified mutations in *ced-5* alleles by determining the sequences of genomic regions produced by amplification with the polymerase chain reaction (PCR).

Generation of CED-5 antibodies and western blot analysis

We used PCR to amplify a region of *ced-5* coding sequence from codon 1,483 to codon 1,750 with plasmid pC5OKBA as a template and oligonucleotides 5'-GGATCCTCATGTTGAAGCCTGATGTGAATG-3' and 5'-GGATCCACTGCTGACAGTCTGCCAAC-3' as primers. The resulting product was cloned into the vector pBluescript II SK+ (Stratagene) via its *Eco*R V site. The insert from the sequence-confirmed construct was then excised using *Bam*H I and cloned into pGEX2T (Pharmacia) and pATH11[31] vectors via their *Bam*H I sites. Both the GST-CED-5 and TrpE-CED-5 fusion proteins were present in inclusion bodies and were purified using standard methods[32]. The GST-CED-5 fusion protein was further purified using 7% SDS-PAGE, excised from the gel and eluted

by electrophoresis. Two rabbits were immunized with gel-purified GST-CED-5 fusion protein. Anti-CED-5 antibodies were affinity-purified from 2 ml antisera by binding to a nitrocellulose filter strip carrying 1 mg TrpE-CED-5 fusion protein and eluting the specifically bound antibodies with 100 mM glycine-HCL (pH 2.5). For western blot analysis, embryonic protein extracts were resolved using 5% SDS-PAGE and transferred to nitrocellulose membranes using standard methods[32]. The blot was incubated with affinity-purified CED-5 antibodies. The ECL detection system (Amersham) was used to detect CED-5 proteins.

Figure 1 Molecular cloning of the *ced-5* gene.

Rescue of the phenotype of persistent cell corpses in *ced-5* mutant animals by germline transformation using genomic DNA clones. The genetic map of the *ced-5* region of chromosome IV is shown above. Cosmid clones and subclones were tested for rescue of the *ced-5* engulfment defect. +, rescue; -, no rescue. Numbers in parentheses indicate the number of rescued lines and the total number of transgenic lines. A partial restriction map of one subclone with *ced-5* rescuing activity is shown. The structure of the *ced-5* gene was deduced by comparing the sequences of genomic DNA and cDNAs. A 5' SL1 *trans*-spliced leader and a 3' poly(A) tail are indicated at the ends of the transcript. Boxes represent exons. Filled boxes indicate the *ced-5* open reading frame; the open box indicates the untranslated region.

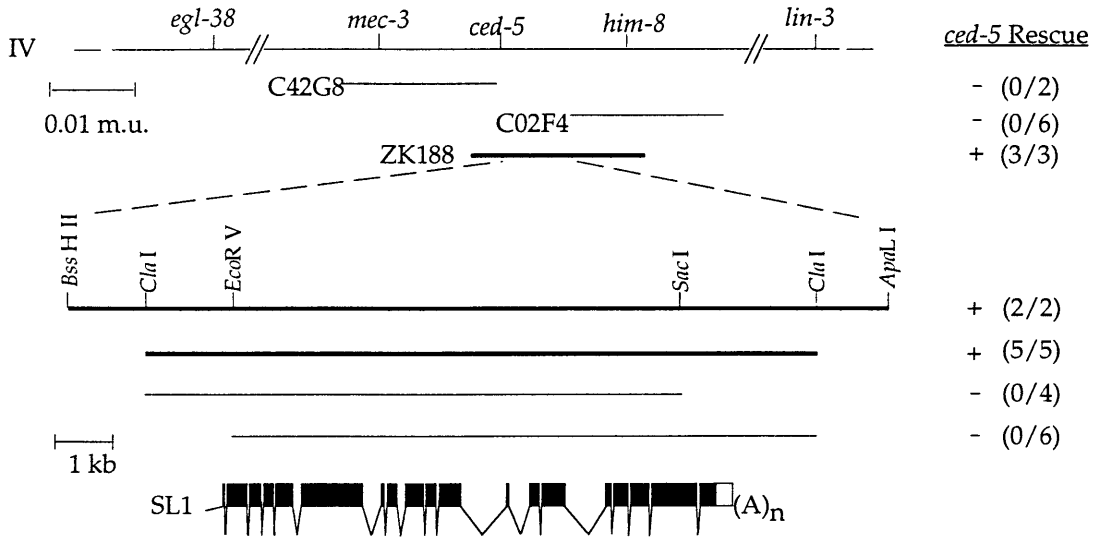

IV
egl-38
mec-3
ced-5
him-8
lin-3
0.01 m.u.
C42G8
C02F4
ZK188
Bss H II
Cla I
EcoR V
Sac I
Cla I
ApaL I
1 kb
SL1
(A)n
ced-5 Rescue
- (0/2)
- (0/6)
+ (3/3)
+ (2/2)
+ (5/5)
- (0/4)
- (0/6)

Figure 2 CED-5 protein sequence and alignment with the human DOCK180 (ref. 5) and *Drosophila* MBC protein[12].

Black boxes indicate amino acids identical between CED-5 and DOCK180 or MBC. Gray boxes indicate amino acids identical only between DOCK180 and MBC. Arrows indicate the positions of nonsense mutations or a frameshift mutation, and vertical bars indicate the positions of splice-site mutations found in *ced-5* mutant alleles. Codon changes are indicated in parentheses.

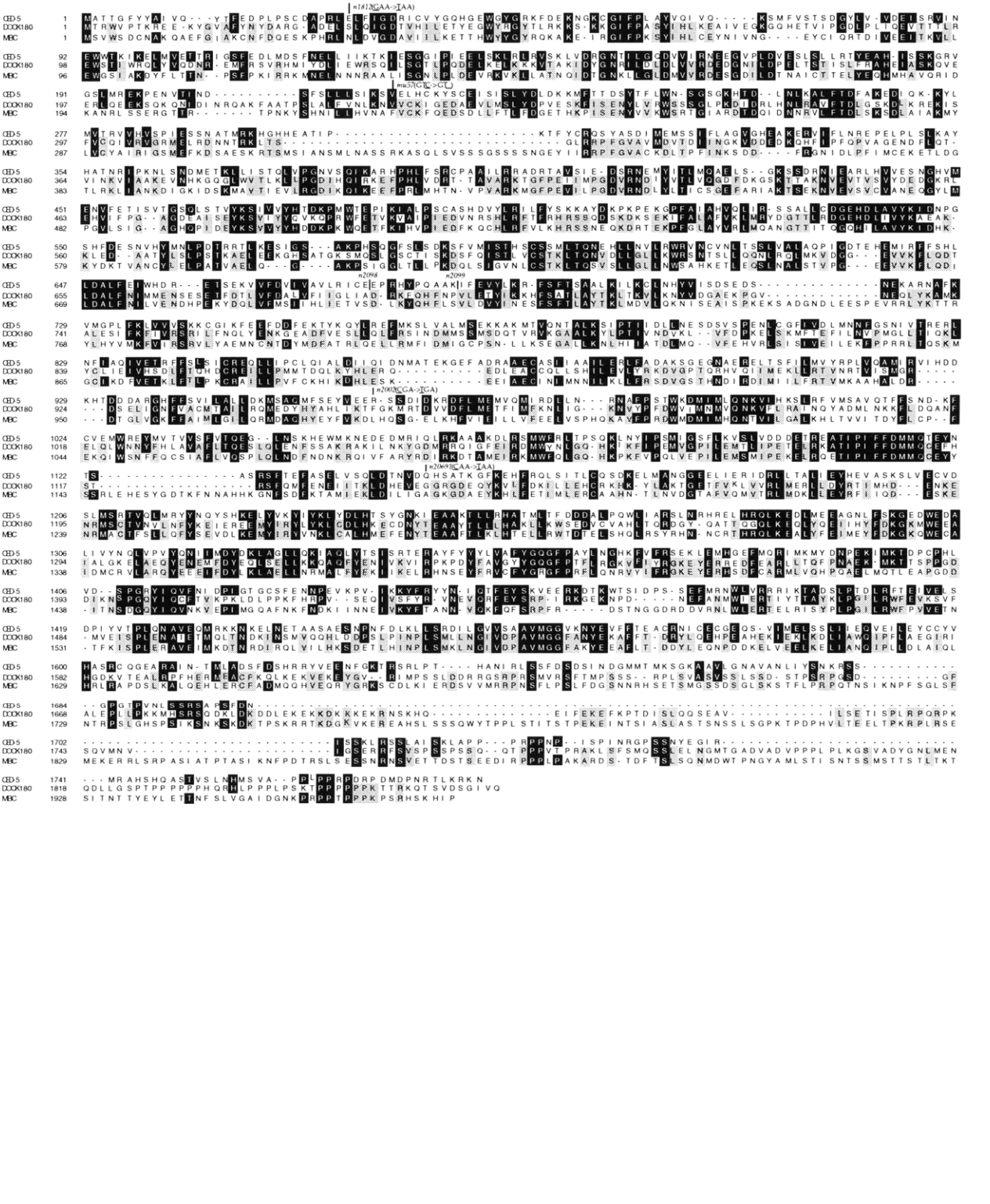

m1412(GAA->TAA)
m3571(TC->GC)
n2098
n2099
n2700(CGA->TGA)
n2069(CAA->TAA)

Figure 3 Western blot analysis of CED-5 protein levels.

We used affinity-purified anti-CED-5 antibodies to probe a blot of embryonic extracts from wild-type, *ced-5(n1812)*, *ced-3(n717)*, *ced-4(n1162)*, and *ced-9(n1950)* animals. 200 μg of protein extracts were loaded in each lane. Equal loading of proteins in each lane was confirmed by Ponceau S staining[32] (data not shown). The sizes of molecular weight markers (High range, Bio-Rad) and the position of the CED-5 protein are indicated.

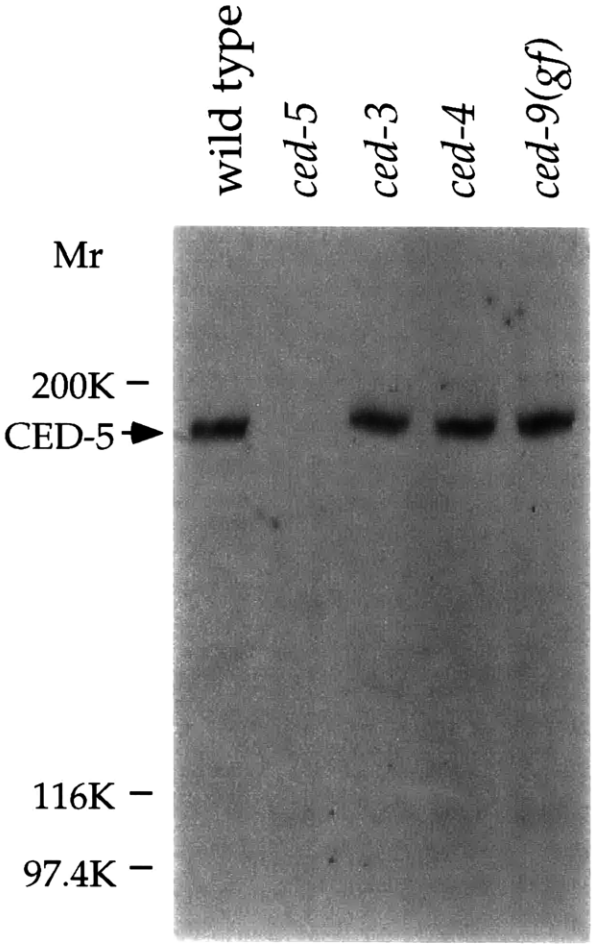
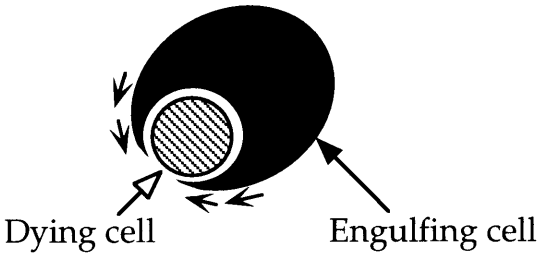

wild type
ced-5
ced-3
ced-4
ced-9(gf)
Mr
200K
CED-5
116K
97.4K

Figure 4 Cell-corpse engulfment and DTC migration are similar processes.

In each case, the surface membrane of a cell (black) extends along the surface of another cell (striped). The small arrows near the black cells indicate the directions of cell-surface extension. Only relevant parts of body muscles are shown.

Engulfment of cell corpses



DTC migration

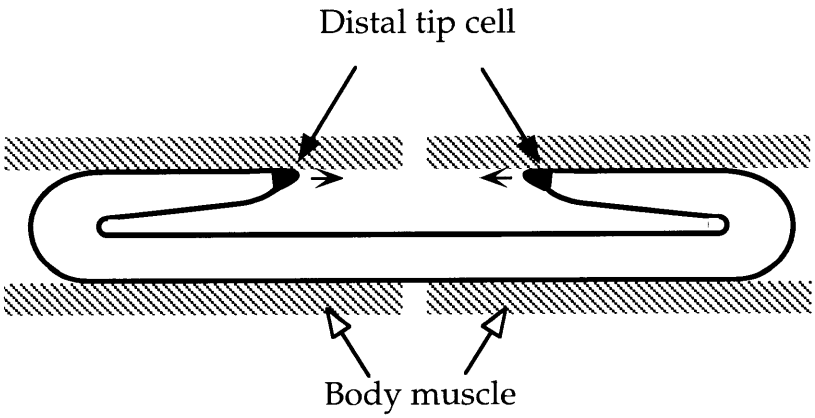


Table 1 *ced-2, 5* and *10* mutants have DTC-migration defects

strain	No. persistent cell corpses* (n=20)	% animals with DTC-migration defect # (n=100)
wild-type	0 ± 0	0
ced-2	31 ± 4	35
ced-5	36 ± 4	77
ced-10	24 ± 4	36
ced-1	30 ± 4	0
ced-6	32 ± 3	0
ced-7	34 ± 3	0
ced-4; ced-5	0 ± 0	72
ced-9(gf); *ced-5*	0 ± 0	76

* Cell corpses are observed in the head of L1 larvae within 1.5 hr. after animals hatch. The data shown are means ± s.e.m. #DTC-migration defect was scored based upon the shape of the gonad in early adults. The following mutants were used: *ced-1(e1735), ced-2(e1752), ced-4(n1162), ced-5(n1812), ced-6(n2095), ced-7(n1996), ced-9(n1950)* and *ced-10(n1993)*.

Table 2 Characterization of *ced-5* Alleles

Allele	Codon Change*	Codon Position	No. Persistent Cell Corpses#	% Animals with DTC-Migration Defect†
n1812	**G**AA⟶**T**AA	28	36 ± 4	77
mu57	GT**C** ⟶ GT_	216	39 ± 4	50
n2002	**C**GA ⟶ **T**GA	962	35 ± 5	50
n2691	**C**AA ⟶ **T**AA	1145	37 ± 6	47
n2098	a**g**|AGG ⟶ a**a**|AGG	676	25 ± 6	54
n2099	a**g**|ATT ⟶ a**a**|ATT	746	27 ± 5	57

*The codon changes are indicated in 6 *ced-5* alleles. Uppercase letters indicate exon sequences; lowercase letters indicate intron sequences. #The cell corpses in the head of L1 larvae within 1.5 hr. after hatch were counted. Data shown are means ± s.e.m. †The DTC-migration defect was scored based upon the shape of the gonadin early adults.

Table 3 Expression of DOCK180 rescued the DTC-migration defect of *ced-5* mutants

hsp transgene*	*ced-5*	DOCK180	GFP	none
% animals with DTC-migration defect	8 ± 2	29 ± 5	69 ± 4	72 ± 5

*The heat-shock constructs were injected into *ced-5(n1812); unc-76(e911)* animals (see Methods). All data depict results obtained after heat shock. The DTC-migration defect was scored based upon the shape of the gonad in early adults. Wild-type animals (n=181) have no DTC-migration defect following heat shock. The data shown are means ± s.e.m. from at least two stably transmitting lines. At least 80 animals were scored from each line. GFP, green fluorescent protein[17].

Table 4 Expression of a *ced-5* cDNA rescued the engulfment defect of *ced-5* mutants

hsp transgene*	heat shock	No. cell corpses in head†					No. cell corpses in germline#
		4-fold embryo	L1	L2	L3	L4	
ced-5	+	0.8 ± 2	6 ± 4	3 ± 2	3 ± 2	2 ± 2	4 ± 5
	−	34 ± 2	30 ± 5	27 ± 3	21 ± 4	15 ± 3	27 ± 4
GFP	+	33 ± 2	30 ± 3	28 ± 2	20 ± 2	16 ± 4	24 ± 6

*The heat-shock constructs were injected into *ced-5(n1812); unc-76(e911)* animals (see Methods). Mixed-staged transgenic progeny were subjected to heat shock (+) or left at 20°C (-).

†Transgenic animals were scored for the number of head cell corpses, which were generated during embryogenesis, and for developmental stage 10 hr. following heat shock.

#Number of cell corpses in each gonadal arm of the transgenic animal was scored 24 hr. following heat shock (see Methods). Data shown are means ± s.e.m. from two independent stably transmitting lines. More than 20 animals were scored from each line. GFP, green fluorescent protein[17].

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Correspondence and requests for materials should be addressed to H.R.H. (e-mail: horvitz@mit.edu).

Chapter 3

C. elegans CED-7, a protein similar to ABC transporters, functions in both dying and engulfing cells during cell-corpse engulfment

Summary

The *C. elegans* gene *ced-7* is required for the engulfment of cell corpses during programmed cell death. Here we describe the molecular and functional characterization of this gene. *ced-7* encodes a protein with sequence similarity to ABC transporters. CED-7 protein is broadly expressed during embryogenesis and is localized to the plasma membrane. Mosaic analysis of *ced-7* shows that *ced-7* functions in both dying cells and engulfing cells during the engulfment of cell corpses. We propose that CED-7 functions to translocate molecules mediating the interaction between cell surfaces of the dying and engulfing cells during engulfment. Like CED-7, the mammalian ABC transporter ABC1 has been implicated in the engulfment of cell corpses, suggesting that CED-7 and ABC1 may be functionally similar and that the molecular mechanism underlying cell-corpse engulfment may be conserved from nematodes to mammals.

Introduction

Programmed cell death is an important cellular process in the development and homeostasis (reviewed by Ellis et al., 1991b; Steller, 1995; Jacobson et al., 1997) . Once cells undergo programmed cell death, their cell corpses are swiftly engulfed by other cells and degraded (reviewed by Ellis et al., 1991b; Savill et al., 1993). Cell-corpse engulfment is a multi-step process, which involves the recognition of a dying cell followed by the extension of pseudopodia and the envelopment of the dying cell by an engulfing cell. Studies in vertebrates have identified a number of molecules that may participate in the recognition step (reviewed by Savill et al., 1993; Hart et al., 1996; Savill, 1997). The exposure of phosphatidylserine on the cell surfaces of dying cells may act as a trigger for their recognition by macrophages (Fadok et al., 1992a; Fadok et al., 1992b). The lectin-like proteins (Duvall et al., 1985) and adhesion molecules, such as vitronectin (Savill et al., 1990; Fadok et al., 1992b) and CD36 (Savill et al., 1991; Savill et al., 1992), on the surfaces of macrophages have been implicated in the recognition of specific carbohydrates and charge-sensitive moieties on the surfaces of dying cells, respectively. However, the mechanism of cell-corpse engulfment remains largely unknown.

In the nematode *C. elegans*, 131 of 1090 somatic cells generated during hermaphrodite development undergo programmed cell death (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). Genetic studies have identified at least six genes, *ced-1, ced-2, ced-5, ced-6, ced-7* and *ced-10* (*ced*, cell death abnormal), which control the engulfment of cell corpses (Hedgecock et al., 1983; Ellis et al., 1991a). Mutations in any of these genes block the engulfment of many cell corpses and result in the phenotype of persistent cell corpses, which are readily distinguishable using Nomarski optics by their refractile and button-like morphology. These engulfment genes are also important for the engulfment of cell corpses in germline cell death (M. Hengartner and H.R.H., unpublished results; Hengartner, 1997). Genetic analysis suggests that the six engulfment genes fall into two groups: *ced-1, 6* and *7* in one group and *ced-2, 5* and *10* in the other (Ellis et al., 1991a). Single mutants or double mutants within the same group show weak engulfment defects, whereas double mutants between the two groups show strong engulfment defects. One model consistent with these observations is that the two groups of genes are involved in two distinct but partially redundant pathways in the engulfment process (Ellis et al., 1991).

Of somatic programmed cell deaths, 90% occur during embryogenesis and the remainder occur during early larval development (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). Like *ced-2, 5, 6,* and *10* mutants, the engulfment defect of *ced-7* mutants can be rescued maternally; homozygous *ced-7* embryos from *ced-7*/+ heterozygous mothers show no defect in the engulfment of somatic cell corpses (Ellis et al., 1991a). To understand the mechanism of cell-corpse engulfment, we cloned the *ced-7* gene. In this work, we present the molecular and functional characterization of this gene.

Results

Positional cloning of *ced-7*

The *ced-7* gene was previously localized to the region between *glp-1* and *unc-50* on chromosome III (Ellis et al., 1991a) (Figure 1A). The interval between these two genes corresponds to approximately 1.2 Mb on the physical map (Coulson et al., 1986). To better define the region containing *ced-7*, we mapped *ced-7* with respect to the cloned gene *emb-9* (Guo et al., 1991) and the fragment length polymorphisms (RFLPs), *eP7* (Greenwald et al., 1987) and *stP127* (Williams et al., 1992) (see Experimental Procedures), which were previously

mapped to this region in the polymorphic strain RW7000. The mapping results localized *ced-7* to an approximately 300 kb region between *emb-9* and the RFLP, *eP7* (Figure 1A). We tested 12 overlapping cosmids from this region for their abilities to rescue the persistent cell-corpse phenotype of *ced-7(n1892)* mutants. We found that the cosmid C29C3 was able to rescue the Ced-7 mutant phenotype. By testing subclones from this cosmid, we localized the *ced-7* rescuing activity to an 11 kb fragment (Figure 1B). Further deletions into this fragment from either the right or the left ends abolished its rescuing activity.

***ced-7* Sequence and Mutant alleles**

We used the 11 kb genomic fragment to isolate *ced-7* cDNA clones and defined the 5' end of the *ced-7* message using the RACE (rapid amplification of cDNA ends) method. The sequence of these cDNAs revealed an open reading frame of 1704 amino acids, a 5' SL1 *trans*-spliced leader found at the 5' end of many *C. elegans* transcripts (Krause and Hirsh, 1987) and a 3' poly(A) tract, confirming that we had identified the complete *ced-7* transcription unit. The *ced-7* genomic sequence has been determined by *C. elegans* genomic sequencing project and its exons have been predicted by the Genefinder program (Wilson et al., 1994). Ten of fourteen exons predicted are consistent with those of *ced-7* cDNA. Northern analysis using the *ced-7* cDNA as a probe revealed a single band of 5.8 kb, consistent with the size of the full-length *ced-7* cDNA (data not shown). The expression of the *ced-7* cDNA under the control of *C. elegans* heat-shock promoters (Stringham et al., 1992) rescued the engulfment defect of *ced-7* mutant animals (Table 2), indicating that the *ced-7* cDNA encodes a functional CED-7 protein.

We have found molecular lesions in eight *ced-7* alleles (Table 1), confirming that we have correctly identified the *ced-7* gene. The alleles *n1996* and *n2094* have early nonsense mutations, presumably deleting more than 90% of CED-7 protein and have no detectable CED-7 protein expression as assayed by western blot analysis or antibody staining (Figure 5; data not shown), consistent with the two alleles being null. One *ced-7* allele, *n1892*, appeared to contain no mutations in the *ced-7* coding sequence and splicing junctions; this allele may contain alterations in the regulatory regions of the gene, since it did not express detectable CED-7 protein as assayed by western blot analysis and antibody staining (data not shown).

***ced-7* encodes a protein with sequence and structural similarity to ABC transporters**

A search of protein databases with the predicted CED-7 protein sequence revealed that CED-7 is similar to ABC (ATP-binding cassette) transporters (Figure 2). Like other members of the ABC transporter superfamily (reviewed by Higgins, 1992; Fath and Kolter, 1993), CED-7 consists of two similar halves. Each half of the protein contains a hydrophobic region with six putative transmembrane domains and a hydrophilic ABC region (Figure 3). The ABC region contains a predicted ATP nucleotide-binding domain (NBD), which includes the Walker A motif ($GX_4GK[S/T]$) (Walker et al., 1982) and Walker B motif (RX_{6-8} hyd_4D) (Mimura et al., 1991), and an ABC signature sequence ([L/Y]SGG[Q/M]), which is diagnostic for members of ABC transporters (Higgins, 1992) (Figures 2 and 3).

ABC transporters have been identified that mediate the transport of a diversity of substrates, ranging from irons, sugars, vitamins, phospholipids, peptides to proteins (Higgins, 1992; Ruetz and Gros, 1994). However, the mechanism by which each ABC transporter achieve its own particular substrate specificity is still poorly understood.

CED-7 is most similar to the ABC1 subfamily of ABC transporters, which includes the mouse ABC1 (Luciani et al., 1994), the mouse ABC2 (Luciani et al., 1994), the human ABC-C (ABC3) (Klugbauer and Hofmann, 1996; Connors et al., 1997), the human ABCR (Allikmets, 1997) and the bovine rim (Illing et al., 1997) proteins. CED-7 is most similar to the ABC-C protein and is 25% and 20% identical to the ABC-C and ABC1 proteins throughout their entire lengths, respectively (Figure 2). The similarity of CED-7 to these two proteins is most striking in the two hydrophilic ABC regions (Figure 2). In addition, members of ABC1 subfamily all have a unique highly hydrophobic domain (HH1) (Luciani et al., 1994) localized between the two halves of the protein (Figures 2 and 3); however, the functional or structural significance of this domain remains to be explored.

While the ABC1 protein is able to transport anions in Xenopus oocytes (Becq et al., 1997), no physiological substrates of the ABC1 subfamily of ABC transporters have been identified. It has been shown that ABC1 is expressed in macrophages and that the ability of macrophages to engulf apoptotic thymocytes, but not yeast cells, was severely impaired when macrophages were loaded with anti-ABC1 antibodies (Luciani and Chimini, 1996). These results suggest that ABC1 may be involved in the engulfment of cell corpses. Therefore, it is possible

that ABC1 and CED-7 may transport similar substrates required for the engulfment process.

The first nucleotide-binding site of CED-7 is more important than the second one for its in vivo function

Two NBDs of some ABC transporters have been shown to be important for substrate transport (Azzaria et al., 1989; Berkower and Michaelis, 1991). Crystallographic and NMR studies of adenylate kinase have suggested that the lysine residue of the Walker A motif interacts with the phosphate group of the bound ATP (Pai et al., 1977; Fry et al., 1988; Saraste et al., 1990) and is important for ATP hydrolysis (Saraste et al., 1990). Mutations that change the conserved lysine residues to arginine in one or both NBDs of the ABC transporter MDR1 disrupted its drug-transport activity, but not its ability to bind the ATP analog, 8-azido ATP, suggesting that such mutations in NBDs do not cause any overt change in protein conformation and the defect of mutant MDR1 proteins in substrate transport may be attributed to the impairment in ATP-hydrolysis (Azzaria et al., 1989). To assess the functional importance of the NBDs for *ced-7* activity and to examine if the two NBDs function equivalently, we mutated the conserved lysine residues to arginine in the first (K586R), the second (K1417R) or both (K586R, K1417R) NBDs and generated *ced-7(n1996)* transgenic animals expressing either the wild-type or mutant CED-7 proteins under the control of the endogenous *ced-7* promoter, P*ced-7*. We then determined the extent of rescue of the *ced-7* engulfment defect by these transgenes (see Experimental Procedures). We found that CED-7 (K1417R) still retained partial rescuing activity, whereas CED-7(K586R) and CED-7(K586R, K1417R) failed to rescue the *ced-7* engulfment defect (Table 2). The expression of CED-7 proteins by these transgenic animals was confirmed by anti-CED-7 antibody staining (data not shown). These results suggest that the first NBD plays a more important functional role for *ced-7* activity than the second NBD.

CED-7 activity is required in both dying cells and engulfing cells during cell-corpse engulfment

Because the engulfment process requires the interaction between a dying cell and an engulfing cell, it is crucial to identify in which cells *ced-7* activity is required. To investigate this issue, we have analyzed *ced-7* genetic mosaics (see Experimental Procedures). Briefly, we used a strain that is mutant for *ced-7* and the cell-autonomous marker *ncl-1* (Hedgecock and Herman, 1995) and carries wild-type copies of *ced-7* and *ncl-1* on a small extrachromosomal duplication of

chromosome III, *qDp3*. Since mutations in *ced-7* and *ncl-1* genes are recessive, animals carrying *qDp3* are generally wild-type. However, *qDp3* is mitotically unstable and is occasionally lost during embryonic cell divisions. Such mitotic loss will generate a clone of genetically mutant *ced-7*(-) *ncl-1*(-) cells recognizable by their enlarged nucleoli phenotype (Ncl) by Nomarski optics in an otherwise genetically *ced-7*(+) *ncl-1*(+) background.

We used these genetic mosaics to analyze the role of *ced-7* in germline cell death. Germline cell corpses are engulfed by gonadal sheath cells, which contact the germline (Riddle et al., 1997; M. Hengartner, E. Hartwieg and H.R.H., unpublished observations). One advantage of choosing to analyze germline cell deaths over somatic cell deaths is that the *ced-7* engulfment defect in germline cell death can not be maternally rescued (data not shown) and therefore the engulfment phenotypes of mosaic animals are not obscured by the maternal product from the wild-type *ced-7* gene on *qDp3* .

The germline in both gonadal arms of adult hermaphrodites is derived from the P3 lineage, while the sheath cells of the anterior and posterior arms are derived from two different blastomeres, MSp and MSa, respectively (Kimble and Hirsh, 1979; Sulston et al., 1983) (Figure 4). We identified candidate mosaics in which *qDp3* has been lost in either the germline or gonadal sheath cells by screening for animals with Ncl cells in P3, MSp, or MSa lineages (Figure 4; see Experimental Procedures). To identify any cells where *ced-7* is dispensable, we also screened for animals with Ncl cell clones that are not lineally related to the germline or the gonadal sheath cells. If *ced-7* function is required in dying cells, the loss of *qDp3* in the germline, but not in other cells, would result in persistent cell corpses in the germline. If *ced-7* function, by contrast, is required in engulfing cells, the loss of *qDp3* in the gonadal sheath cells, but not in other cells, would result in persistent cell corpses in the germline. On the other hand, if *ced-7* function is required in both dying cells and engulfing cells, the loss of *qDp3* in either germline or gonadal sheath cells would result in persistent cell corpses.

We identified 56 mosaic animals in our screen (Figure 4). Nine animals lost *qDp3* in the sheath cells of the anterior gonadal arms and are Ced-7 in these arms but are wild-type in the posterior arms. Similarly, ten animals lost *qDp3* in the sheath cells of the posterior gonadal arms and are Ced-7 in these arms but are wild-type in the anterior arms. 5 animals lost *qDp3* in sheath cells of both gonadal arms and are Ced-7 in both arms. In all cases the Ced-7 gonadal arms displayed on average 28 cell corpses, approximately the same number observed

in *ced-7(n1892)* mutants; this finding indicates that *ced-7* function is required in the engulfing gonadal sheath cells. In addition, 5 mosaic animals in which *qDp3* was lost in the germline, also showed the Ced-7 phenotype in both gonadal arms with an average 15 cell corpses. This observation indicates that *ced-7* function is also important in dying cells during the engulfment process. However, in 27 mosaic animals in which *qDp3* was lost in the cells other than germline or gonadal sheath cells, we observed less than 4 cell corpses in the gonadal arms as in those of wild-type animals. This observation is consistent with the interpretation that *ced-7* function is dispensable in the cells other than dying and engulfing cells during cell-corpse engulfment.

CED-7 is widely expressed and is localized to the plasma membrane during embryogenesis

We raised polyclonal antibodies against recombinant CED-7 protein (see Experimental Procedures). Using the affinity-purified CED-7 antibodies, we detected by western blot analysis a protein band of approximate molecular mass 170K from wild-type embryonic extracts, which is slightly smaller than the size predicated from the CED-7 amino acid sequence (191 K) (Figure 5). This protein was absent in extracts of the *ced-7(n1996)* mutant (Figure 5), indicating that it is indeed the product of the *ced-7* gene.

We used the purified CED-7 antibodies to stain animals. We found that CED-7 was widely expressed in embryos and was localized to the plasma membrane (Figure 6A). In larvae and adults CED-7 expression appeared restricted to specific cells. CED-7 was detected in the amphid sheath cells, pharyngeal-intestinal valve (Figure 6B) and phasmid sheath cells (Figure 6C). CED-7 expression was also detected in both germline precursors and germline except sperms in larvae and adults (Figure 6D), respectively; this observation is consistent with the requirement of *ced-7* function in the germline during germline cell death. *ced-7(n1996)* and *ced-7(n2094)* mutant animals lacked any somatic staining, but we occasionally observed staining in oocytes in *ced-7(n1996)* and *ced-7(n2094)* mutant animals and therefore the oocyte staining may not be specific. The analysis of *ced-7* genetic mosaics showed that *ced-7* function is required in the gonadal sheath cells; however, we could not detect CED-7 expression in these cells. We believe that in these cells CED-7 protein is expressed at levels below which it can be detected by the anti-CED-7 antibodies.

We also examined the CED-7 expression pattern in other mutants defective in the engulfment of cell corpses, *ced-1, ced-2, ced-5, ced-6* and *ced-10.*

We found that the CED-7 expression pattern is not altered in these mutants (data not shown). These five engulfment genes therefore do not regulate the expression or localization of the CED-7 protein.

None of the somatic cells where we detected CED-7 expression in larvae or adults appear to be involved in cell-corpse engulfment. We thus examined if *ced-7* has additional roles in these cells. Among these cells, the function of amphid sheath cells is better understood. Amphid sheath cells are supportive cells, which ensheathe chemosensory neurons in a pair of sensory organs called amphids in the head of the animal. The amphids have opening to the outside, such that the chemosensory neurons are exposed to the environment. One of the behaviors mediated by amphids is osmotic avoidance; animals avoid high concentrations of a number of sugars and salts (Culotti and Russell, 1978). We found that *ced-7(1996)* and *ced-7(2094)* mutant animals behave indistinguishably from wild-type animals in osmotic avoidance assays (data not shown), indicating that the amphid sheath cells of these *ced-7* mutant animals are at least partially functional. In addition, we also found no obvious ultrastructural defects in the amphid sheath cells and the pharyngeal-intestinal valves of *ced-7(1996)* and *ced-7(2094)* mutants in electron micrographs (data not shown). Therefore, the CED-7 expression in amphid sheath cells and pharyngeal-intestinal valves may not be essential for the function or gross cellular structures of these cells.

Discussion

One prominent feature of programmed cell death is the engulfment of dying cells. We showed that the *C. elegans ced-7* gene, which is required for the engulfment process, encodes a protein with sequence similarity to ABC transporters. Like other members of the ABC transporter superfamily, the CED-7 protein has two NBDs. The first NBD appears to be more important than the second one, as the mutation of the conserved Walker lysine residue in the first NBD has a more severe effect on *ced-7* activity than that of the second NBD. Similar results were obtained in the case of CFTR (cystic fibrosis transmembrane conductance regulator), another member of the ABC transporter superfamily. The analysis of the mutant CFTRs revealed that the two NBDs have distinct roles in controlling CFTR channel activity (Carson et al., 1995). The first NBD appears to control the channel opening, whereas the second NBD seems to control the channel closing once the channel opens (Carson et al., 1995). The differential

importance of the two NBDs of the CED-7 protein may therefore indicate the mechanistic difference of the first and the second NBDs for CED-7 function.

One characteristic feature of ABC transporters is unidirectionality of substrate transport (Higgins and Gottesman, 1992; Ruetz and Gros, 1994). This transport process appears to be export, rather than import, in almost all ABC transporters with identified substrates, except CFTR, which acts as a channel (Higgins, 1992; Becq et al., 1997). Recent studies on human MDR1 and MDR3 have shown that these two ABC transporters can function as flippases to translocate lipid from the inner leaflet to the outer leaflet of the plasma membrane (van Helvoort et al., 1996). Such translocation, without the secretion of substrates into the extracellular space, may result in the reorganization of lipid composition, and the redistribution or the modulation of certain cell-surface membrane proteins (Conforti et al., 1990). By analogy, CED-7 could function as a transporter or flippase in the process of engulfment.

We found that CED-7 protein is ubiqitously expressed in embryos, consistent with its role in the engulfment of embryonic cell corpses. With its broad expression pattern, CED-7 activity must be tightly controlled so that the CED-7-mediated engulfment specifically targets dying but not viable cells. Since CED-7 may act as a transporter, as suggested by its sequence, the regulation of CED-7-mediated engulfment may be achieved by controlling either the transporter activity of CED-7 itself or the accessibility of the substrates that CED-7 transports. A Prosite search with the CED-7 amino acid sequence reveals several potential phosphorylation sites, suggesting that CED-7 protein activity might be regulated by such covalent-modifications, just as the activity of CFTR is controlled through the phosphorylation by cAMP-dependent protein kinase (PKA) (Cheng et al., 1991).

CED-7 protein is localized to the plasma membrane, consistent with its sequence as an ABC transporter. It also indicates that CED-7 activity may be important for the interaction between the cell surfaces of the dying and engulfing cells. Interaction between the dying and engulfing cells is required in two aspects of the engulfment process: the recognition process, which triggers phagocytosis, and the adhesion process, as the engulfing cell extends pseudopodia around the dying cell during phagocytosis.

But how may CED-7 function as an ABC transporter to mediate recognition or adhesion? Given that *ced-7* function is required in both dying and engulfing cells, if CED-7 on both cells translocate identical substrates, the

substrates are probably not for marking dying cells for recognition. Instead, the substrates may modulate the membrane properties of both the dying cell and the engulfing cell to facilitate their adhesion during phagocytosis. However, we could not rule out the possibility that CED-7 might translocate different molecules in dying and engulfing cells, given by the findings that some ABC transporters can transport different hydrophobic molecules (Higgins, 1992). Future work on the identification and the characterization of CED-7 substrates should help to understand the molecular mechanism of CED-7 function.

Like CED-7, ABC1 has been implicated to act in macrophages during the phagocytosis of apoptotic cells in mammals (Luciani and Chimini, 1996), although the possibility that ABC1 might also play a role in the dying cell remains to be explored. The sequence and potential functional similarity of CED-7 and ABC1 suggests that these two proteins might be homologs. If so, the process of cell-corpse engulfment in which *ced-7* acts may have been conserved through evolution, and there may well be a common molecular mechanism responsible for the engulfment of cell corpses in all metazoans.

Experimental Procedures

Strains

All strains were grown at 20°C, except where noted. All mutations were generated in a Bristol N2 background, which we used as the standard wild-type strain. The following mutations were used: LGIII *ced-4(n1162), unc-86(n946), emb-9(hc70), ced-7(n1892, n1996, n1997, n1998, n2001, n2094, n2690, n3072* and *n3073), ced-9(n1950), unc-50(e306)*; LGIV *ced-3(n717);* LGV *unc-76(e911)* and wild polymorphic strain: RW7000. *emb-9(hc70)* was obtained from the Caenorhabditis Genetics Center.

Mapping *ced-7*

We showed that *ced-7* lies between *emb-9* and *unc-50* by four-factor mapping: 22/56 Unc-86 non-Ced non-Unc-50, 28/56 non-Unc-86 non-Ced Unc-50, 0/56 Unc-86 Ced non-Unc-50 and 6/56 non-Unc-86 Ced-7 Unc-50 recombinants from *unc-86 ced-7(n1892) unc-50/emb-9* heterozygotes segregated *emb-9*.

We determined the position of *ced-7* with respect to the RFLPs *ep7* and *stp127* as described by Ruvkun et al. (1989). In brief, we obtained N2-RW7000 recombinants in the *ced-7* region by mating *unc-86 ced-7(n1892) unc-50/+++* males with RW7000 hermaphrodites to generate *unc-86 ced-7 unc-50*(N2)/+++(RW7000)

heterozygotes. From these animals, Unc-86 non-Unc-50 and non-Unc-86 Unc-50 recombinants were picked. Progeny homozygous for each recombinant chromosome were maintained and their genotypes at *ep7* and *stp127* loci were determined by genomic southern blots. The distances, given in recombinant chromosomes per total chromosomes examined, were *unc-86*(9/18)*ced-7*(1/18)*eP7*(5/18)*stP127* (3/18)*unc-50*.

Transgenic animals

For the genomic rescue experiments, we injected DNAs into *ced-7(n1892)* animals at concentrations of 25-50 μg/ml with the dominant roller marker pRF4 (50 μg/ml), as previously described (Mello et al., 1992). To determine the extent of rescue, we counted the cell corpses in the head of four-fold embryos from the stably transmitting lines, using Nomarski optics, as previously described (Ellis et al., 1991a). Non-rescued embryos have about 34 corpses. Embryos with 0-5 corpses were scored as rescued for the *ced-7* engulfment defect.

For the NBD analysis, we co-injected DNAs at concentrations of 50 μg/ml into *ced-7(n1996); unc-76(e911)* with the *unc-76* rescuing plasmid p76-16B (Bloom and Horvitz, 1997) to establish transgenic lines and the *egl-5*::GFP plasmid pSC212 (A. Chisholm and H.R.H., unpublished results) to identify transgenic embryos as transformation markers (50 μg/ml each).

Plasmid Construction

To make the mutant *ced-7*(K586R) cDNA construct, we first introduced a Lys^{586}->Arg change (K586R) in the first NBD by the polymerase chain reaction (PCR) using the plasmid p83.c7 as a template and the oligonucleotides c7r1atp, CTCGAGAAGGTTGTACTACGACCAGCTCC and c71tm2, AGAATTCCATCGAGCCCTCC as primers. To make the mutant *ced-7*(K1417R) cDNA, we introduced a Lys^{1417}->Arg change (K1417R) in the second NBD by PCR using the primers c7r2atp, ACCGGTTAAAATATTGAATGTTGTAGTACGTCCAGCTCC and c72tmp2, TGCCACCAGCCACAATTGGA. The resulting PCR products were cloned to the pBluescript SK+ vector (Stratagene) at its *Eco*R V site.

The sequence-confirmed constructs were cut with *Bgl* II and *Xho* I for *ced-7*(K586R) and with *Sna*B I for *ced-7*(K1417R) PCR products and the DNA fragments were cloned to the p83.c7 plasmid previously cut with appropriate enzymes to generate p83.c7(K586R) and p83.c7(K1417R) constructs, respectively. To generate the p83.c7(K586R, K1417R) construct, we excised the *Sna*B I fragment from p83.c7(K1417R) and inserted the fragment into P83.c7(K586R) constructs via

the *Sna*B I sites. To construct P_{ced-7} *ced-7* and its mutant variants, we excised p83.c7 and its mutant derivatives with *Bgl*II-*Hpa* I and cloned the fragments into the *ced-7*-rescuing plasmid pC7KEN, which contains the 11 kb *ced-7* genomic fragment, previously cut with *Bgl* II and *Hpa* I.

Antibodies and immunostaining

We PCR amplified a region of *ced-7* coding sequence from codon 1339 to codon 1704 with the plasmid p83.c7 as a template and the oligonucleotides, GGAGATCTTGGACTGTGCGTCGATCT and CCAGATCTTCAGACATGTGGAATGG as primers. The resulting 1.1 kb product was cut with *Bgl*II and cloned into pGEX-2T (Pharmacia) and pATH11 E. coli expression vectors (Rimm and Pollard, 1989) via their *Bam*HI sites. Both GST-CED-7 and TrpE-CED-7 fusion proteins were present in the inclusion bodies and were purified using standard methods. The GST-CED-7 fusion protein was further purified using 7% SDS-PAGE. The correct band was excised from the gel following visualization by soaking gels in 0.3 M CuCl2 and electroeluted. Gel-purified CED-7 protein was mixed with RAS adjuvant (RIBI ImmunoChem Research) in PBS and injected into two rabbits at a dose of 0.4-1 mg of protein per injection. The anti-CED-7 antibodies were purified by binding to a nitrocellulose filter strip carrying TrpE-CED-7 fusion protein and eluting the specifically bound antibodies with 100 mM glycine-HCl (pH 2.5).

For western blot analysis, embryos and worms were washed off plates with dH_2O and treated with hypochlorite to obtain embryos. The embryos were then sonicated in 1X SDS sample buffer and the extracts were fractionated using 5% SDS-PAGE and transferred to nitrocellulose membranes. The CED-7 protein was visualized using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and chemiluminescent detection reagents (Amersham).

For immunofluorescence detection of CED-7, embryos were fixed as described by Guenther et al. (1996) and larvae and adults were fixed as previously described by Finney et al. (1990). The fixed animals were stained with a 1:50 dilution of purified CED-7 antibodies at 4°C overnight, washed three times with PBST-B (1XPBS, 0.1% BSA, 0.5% Triton-X-100, 1 mM EDTA), followed by an incubation with a 1:50 dilution of FITC conjugated goat anti-rabbit IgG (Cappel) at 37°C for 2 hours and washed as before. Stained worms were mounted in 1% DABCO in 70% glycerol and visualized using a Bio-Rad MRC-500 confocal microscope.

Analysis of *ced-7* Genetic Mosaics

We analyzed *ced-7* genetic mosaics using the strain MT9149, *ncl-1(e1865) unc-36(e251) ced-7(n1892); qDp3(ncl-1(+) unc-36(+) ced-7(+))*. The Unc-36 phenotype is produced if *qDp3* is lost in Po, AB or ABp (Kenyon, 1986).

We raised animals at 25°C, since this temperature was reported to increase slightly the frequency of *qDp3* mitotic loss (Clark et al., 1993). We used Nomarski optics to screen L4 non-Unc animals from the strain MT9149 for mosaic animals in which some, but not all, cells lost the duplication *qDp3* and were Ncl-1. We scored some or all of the following cells to determine the loss points. Loss points and cells scored (in parenthesis) were: MSaa (m3DL, m4DL, I3 and I4), MSapa (muscle and mid-body ceolomocytes), MSappp (head muscle), MSappap (head muscle), MSpa (m3DR, m4DR and M4), MSppa (head muscle and anterior ceolomocytes), MSpppp (head muscle), MSpppap (head muscle), C (mid-body muscle), D (head and anterior body muscle) and AB (m3L, m3VL, m3R, m4R and excretory canal). We identified 50 mosaic animals out of 3050 nonUnc animals screened. Since we only isolated one mosaic animal in which *qDp3* was lost in the germline lineage, we screened an additional 9935 L4 nonUnc progeny and focused only on identifying such mosaic animals. Since the Ncl phenotype can not be reliably scored in the germline, we isolated mosaic animals which had lost *qDp3* in the D lineage and generated only Unc progeny to ensure that *qDp3* was lost in the germline. We isolated four mosaic animals in this way. To investigate if *ced-7* function is dispensable in the AB lineage, we also scored L4 Unc progeny with nonNcl cells in the P1 lineage. Such animals would have lost *qDp3* in AB or ABp lineages. Out of about 6000 animals scored, we isolated two mosaic animals.

All L4 mosaic animals identified were isolated from slides and transferred to Petri dishes at 20°C for recovery. After allowing the animals to recover for 48 hrs, we scored the number of cell corpses and the Ncl-1 phenotype of the gonadal sheath cells in each gonadal arm of the animals using Nomarski optics. The presence of *qDp3* in P4 (the germline) was assessed by scoring for phenotypically wild-type progeny. In almost all cases, the duplication loss in the mosaic animals isolated can be accounted for the loss at a single mitosis point.

Figure 1 Molecular cloning of the *ced-7* gene.
A, The genetic map near the *ced-7* locus on chromosome III is shown above. The cosmid clones shown below were tested for their abilities to rescue the *ced-7* engulfment defect. The cosmid C29C3 in bold is able to rescue the defect.
B, A partial restriction map of one subclone of the C29C3 cosmid with *ced-7* rescuing activity is shown. The fragments derived from this subclone were used to define the minimal region containing the *ced-7* rescuing activity by the germline transformation experiment. +, rescue; -, no rescue. Numbers in parentheses indicate the number of rescued lines and the total number of transgenic lines. The structure of the *ced-5* gene was deduced by comparing the sequences of genomic DNA and cDNAs. A 5' SL1 *trans*-spliced leader is indicated. Boxes represent exons. Filled boxes indicate the *ced-5* open reading frame; open boxes indicates the untranslated region. The transcription direction goes from the right to the left.

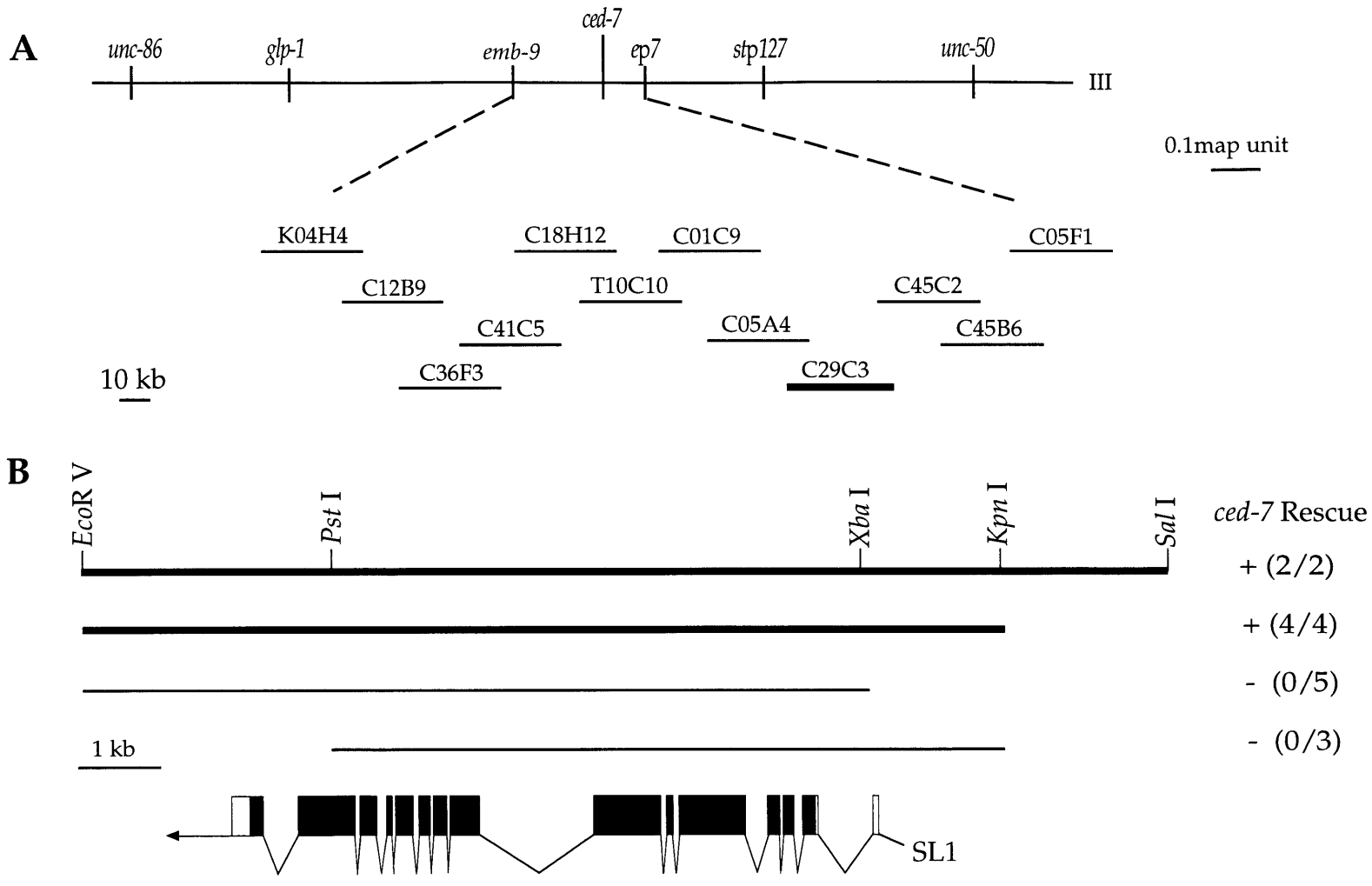

A
unc-86
glp-1
emb-9
ced-7
ep7
stp127
unc-50
III
0.1map unit
K04H4
C18H12
C01C9
C05F1
C12B9
T10C10
C45C2
C41C5
C05A4
C45B6
C36F3
C29C3
10 kb
B
EcoR V
Pst I
Xba I
Kpn I
Sal I
ced-7 Rescue
+ (2/2)
+ (4/4)
- (0/5)
- (0/3)
1 kb
SL1

Figure 2 CED-7 protein sequence and alignment with the human ABC-3 and the mouse ABC1.

CED-7 protein sequence and alignment with the human ABC-3 (Connors et al., 1997) and the mouse ABC1 (Luciani et al., 1994) proteins. Black boxes indicate amino acids identical between CED-7 and ABC-3 or ABC1. Gray boxes indicate amino acids identical only between ABC-3 and ABC1. The Walker A and Walker B motifs of the nucleotide-binding domains, the ABC signature sequence (SS) and the highly hydrophobic domain (HH1) are indicated.

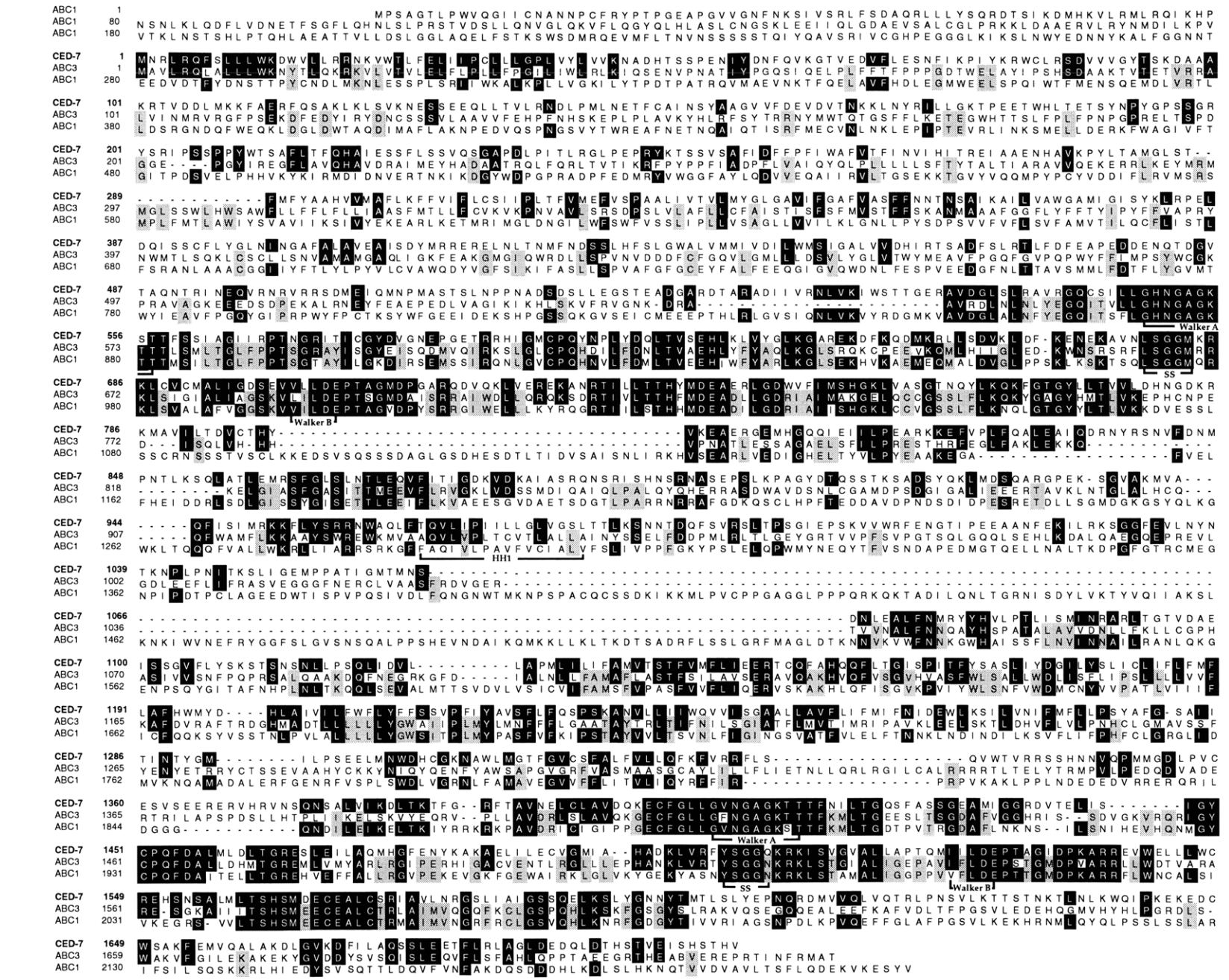
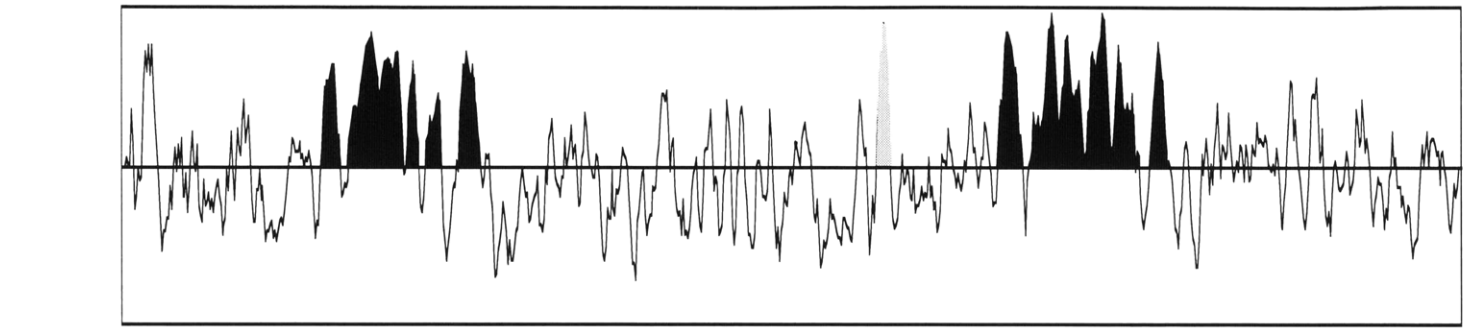

A
ABC1
CED-7
ABC3
Walker A
Walker B
SS
HH1

Figure 3 The potential domain organization of CED-7.
A, hydropathy profile of CED-7. The plot is generated using the algorithm and hydrophobicity values of Kyte and Doolittle (1982) for a window size of 11 residues. The hydrophobic and hydrophilic regions are above and below the central line, respectively. The 12 potential transmembrane domains are marked in black and the HH1 domain is marked in gray.
B, The schematic drawing of CED-7, which shows the relative positions of the transmembrane domains, the HH1 domain and the nucleotide-binding domains (NBD) marked in striped boxes. The first and the second striped boxes of the NBD indicate the predicted Walker A and Walker B motifs, respectively.

B



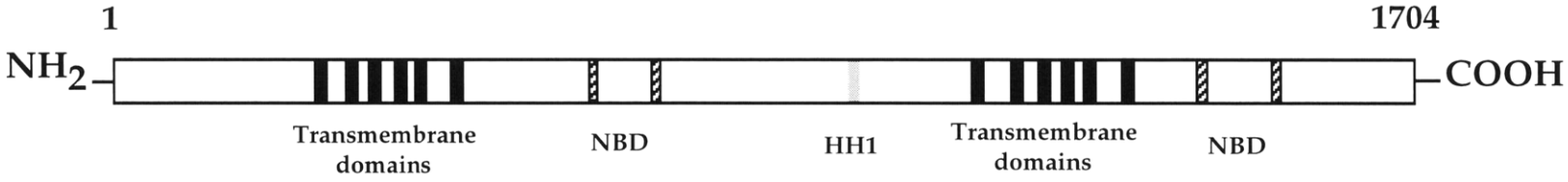
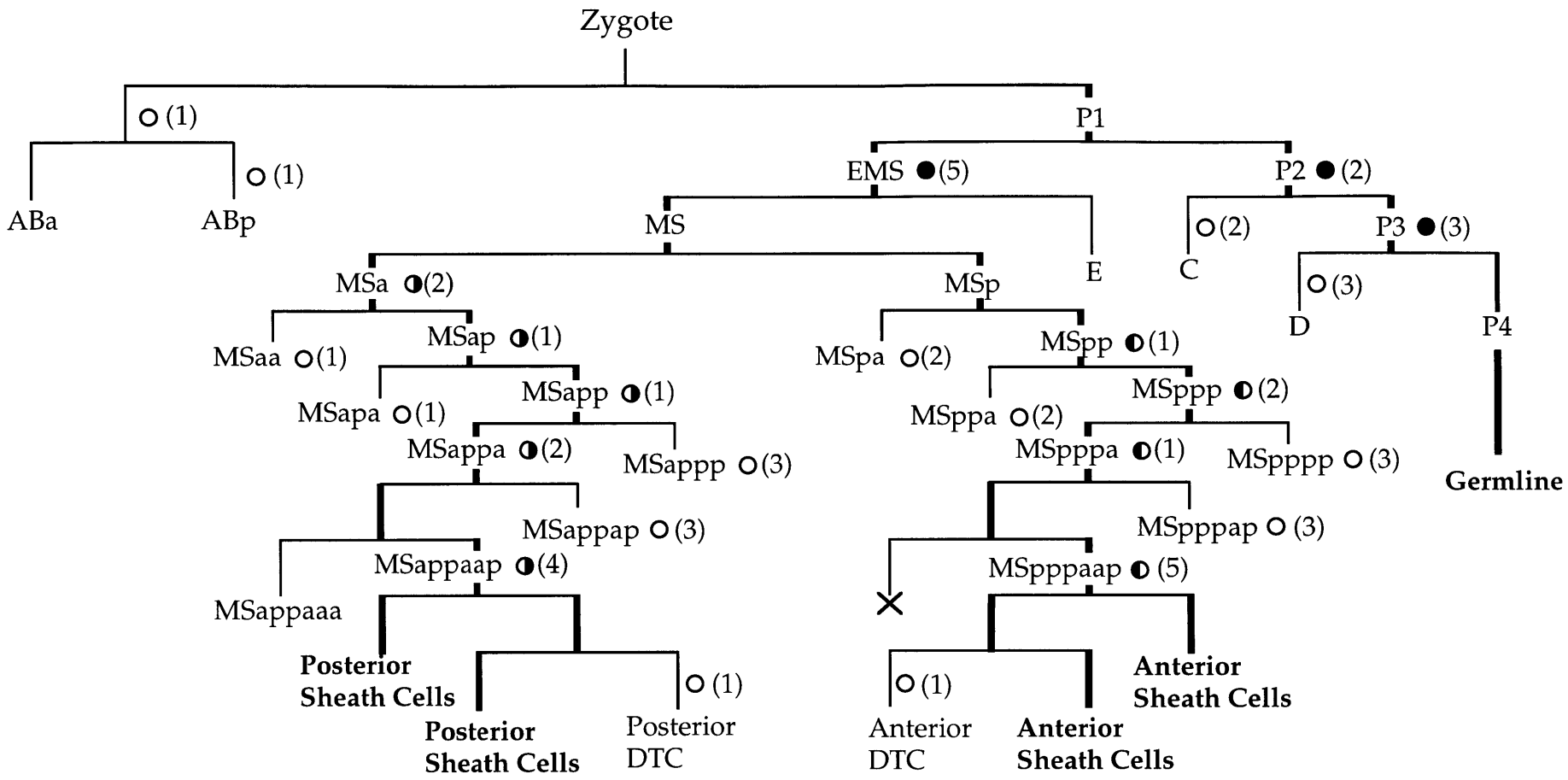

1
1704
NH2
COOH
Transmembrane domains
NBD
HH1
Transmembrane domains
NBD

Figure 4 *ced-7* mosaic analysis.
The partial cell lineage adapted from Sulston et al (1983) and Kimble et al (1979) is shown. The lineages that give rise to the germline or the gonadal sheath cells of the anterior or posterior gonadal arms are indicated in thick vertical bars. The phenotypic symbols for each class of mosaic animals were positioned to the mitosis point at which the duplication was lost and the number of such mosaic animals scored were followed in parenthesis. The Ncl phenotype in P4 and E lineages can not be scored reliably.



Phenotype of genetic mosaic aniamals:

- ● Ced-7 in both gonadal arms
- ○ wild-type in both gonadal arms
- ◐ Ced-7 in the anterior goandal arm and wild-type in the posterior gonadal arm
- ◑ wild-type in the anterior gonadal arm and Ced-7 in the posterior gonadal arm

Figure 5 Western blot analysis of CED-7 protein.
We used affinity-purified anti-CED-7 antibodies to probe a blot of embryonic extracts from wild-type and *ced-7(n1892)* animals. 200 μg of protein extracts were loaded in each lane. The loading of proteins in both lanes was confirmed by Ponceau S staining (data not shown). The sizes of molecular weight markers (High range, Bio-Rad) and the position of the CED-7 protein are indicated.

A

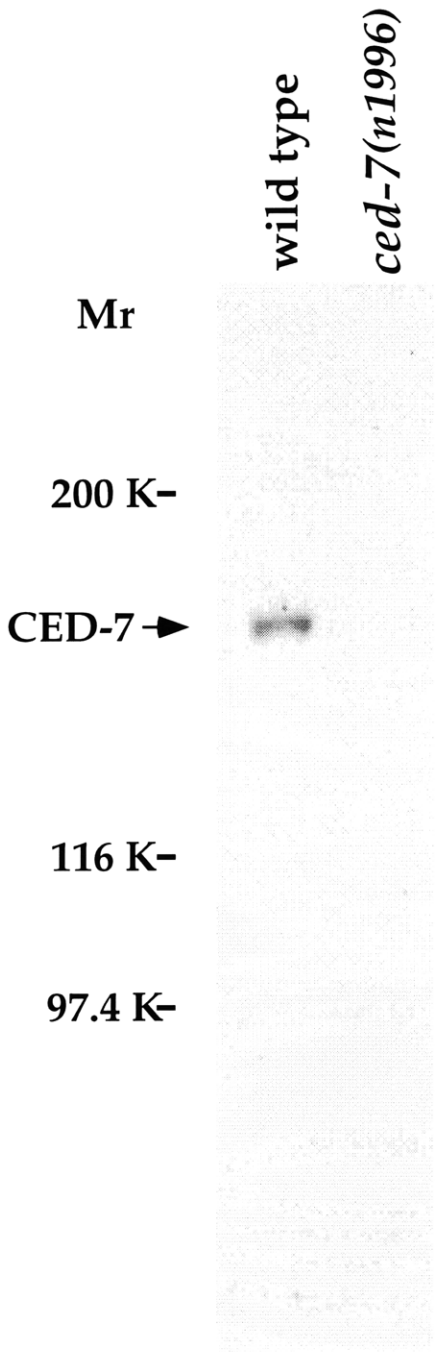
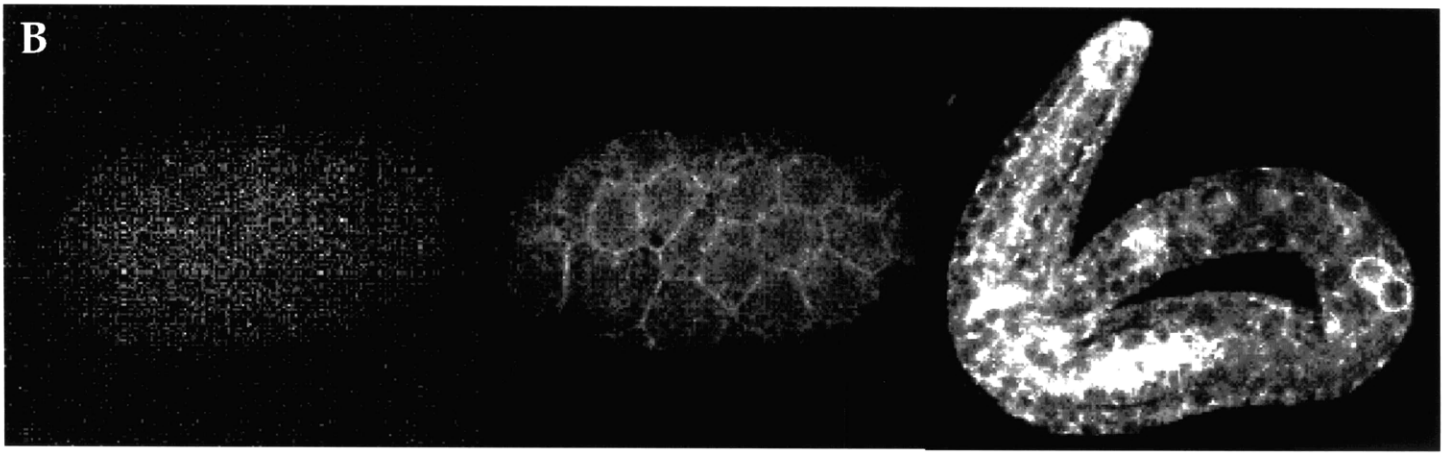
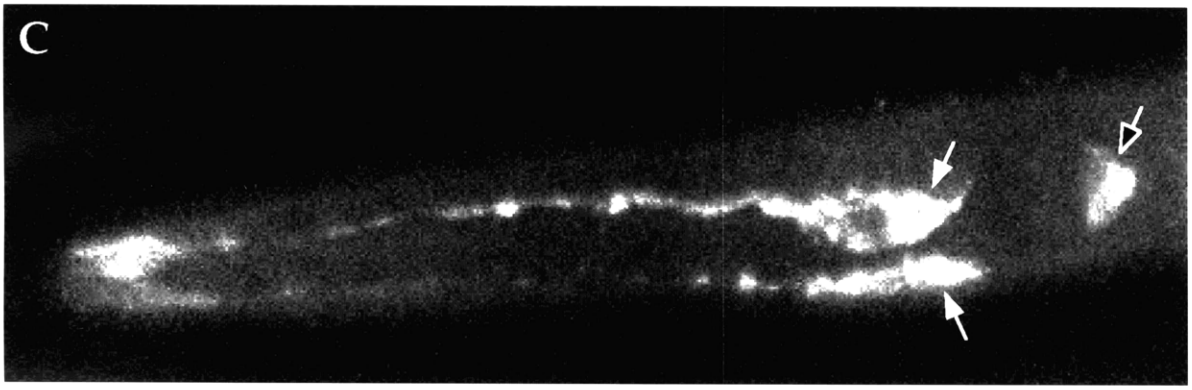
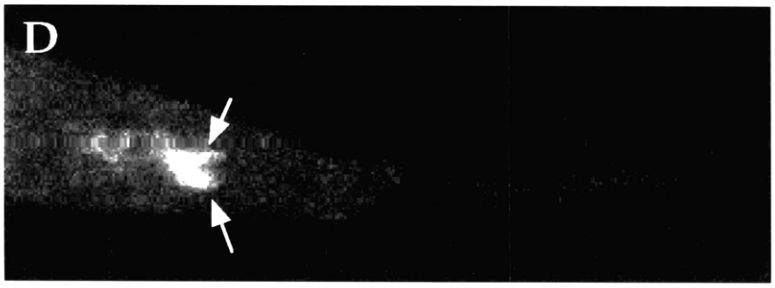


Figure 6 CED-7 protein expression.
A, Anti-CED-7 antibody staining of a *ced-7(n1996)* mutant embryo at 8-cell stage (left), a wil-type embryo at 8-cell stage (center) and a wild-type embryo at ~558-cell stage (right). The two brightly stained cells on the ventral mid body of the right embryo are Z2 and Z3, the germline precursor cells.
B, Anti-CED-7 antibody staining of the head of a wild-type L1 (first larval stage) hermaphrodite. The pharyngeal-intestinal valve and the cell bodies of a pair of amphid sheath cells are indicated by open and filled arrows, respectively. The anterior is to the left. The picture is the projection of 4 serial confocal images.
C, Anti-CED-7 antibody staining of the tail of a wild-type L1 (first larval stage) hermaphrodite. The phasmid sheath cells are indicated by arrows. The anterior is to the left.
D, Anti-CED-7 antibody staining of the germline of the partial anterior gonadal arm of a wild-type adult hermaphrodite.


B


C


D

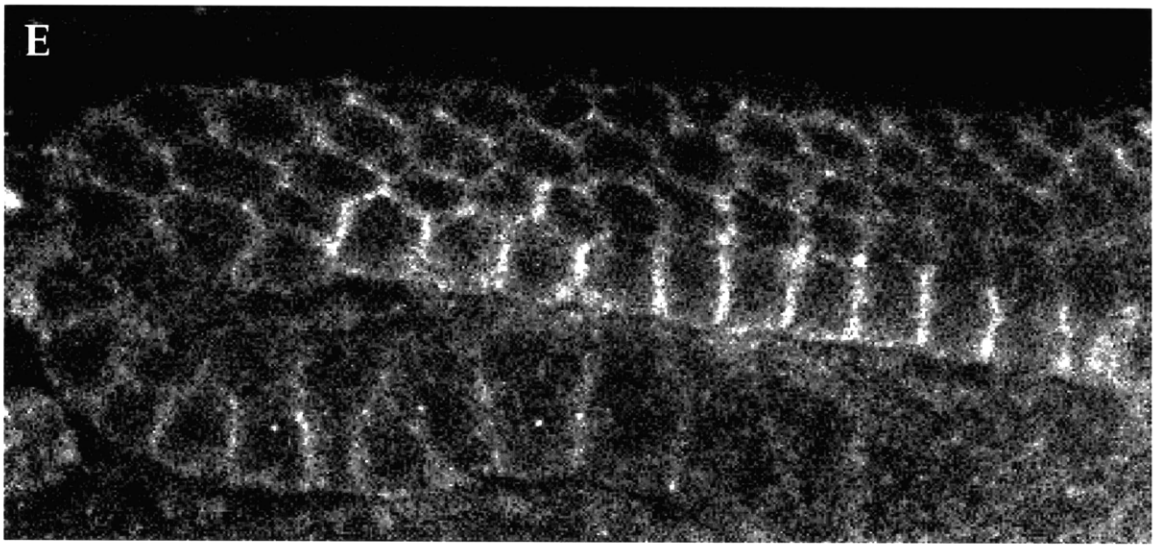

E

Table 2. Structure-function analysis of the CED-7 nucleotide-binding domains

P_{ced-7} Construct[a]	Array[a]	No. persistent corpses (n=15)[b]	Range of persistent corpses[b]
None	-	34 ± 4	29-40
CED-7	1	0 ± 0	0-2
	2	0 ± 0	0-1
	3	0 ± 0	0
CED-7(K586R)	1	37 ± 3	33-43
	2	35 ± 3	30-40
	3	35 ± 4	25-43
CED-7(K1417R)	1	11 ± 6	1-18
	2	21 ± 7	8-36
	3	13 ± 7	1-22
CED-7(K586R, K1417R)	1	35 ± 5	27-45
	2	36 ± 3	32-41
	3	34 ± 3	29-40

[a]The P_{ced-7} constructs were injected into *ced-7(n1996)* mutant animals (see Experimental Procedures). Each array represents an extra-chromosomal transgene carried by a different transgenic line.

[b]Cell corpses were scored in the head of four-fold stage embryos. Mean ± s.e.m.

Table 1. Characterization of *ced-7* alleles

Allele	Nucleic acid and amino acid changes	Codon Position	No. Persistent Corpses (n=20)[b]
n1996	**C**GA (R) ⟶ **T**GA (stop)	5	34 ± 4
n2094	**C**AA (Q) ⟶ **T**AA (stop)	116	36 ± 4
n3072	G**A**A (E) ⟶ G**G**A (G)	639	33 ± 6
n1997	**C**GA (R) ⟶ **T**GA (stop)	1074	34 ± 3
n2690	a**g**|AGT ⟶ a**a**|AGT	1200	16 ± 5
n1998	TG**G** (W) ⟶ TG**A** (stop)	1300	30 ± 3
n3073	**A**GA (R) ⟶ **T**AG (stop)	1332	33 ± 5
n2001	TG**G** (W) ⟶ TG**A** (stop)	1540	32 ± 4
n1892	ND	ND	36 ± 4

[a]We did not identify a mutation in the *ced-7* coding sequence and splice junction sites in *n1892* allele. ND, not determined.

[b]Cell corpses in the head of L1 larvae within 1.5 hr. of hatching were counted. Mean ± s.e.m.

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Appendix
Studies of DNA degradation during programmed cell death in *C. elegans* using TUNEL

One hallmark of programmed cell death (apoptosis) is DNA fragmentation into internucleosomal fragments. However, the regulation and mechanism responsible for DNA degradation is largely unknown. To study this process, we have adapted the TUNEL (TdT-mediated dUTP nick-end labeling) technique for use in *C. elegans*. TUNEL labels DNA ends, which are abundant in dying cells as a consequence of DNA degradation. We found that TUNEL stains only a small subset of dying cells in wild-type embryos. In contrast, *nuc-1* embryos, which are defective in some aspects of DNA degradation, contain many persistent TUNEL-positive signals. We propose that DNA degradation is normally very rapid in dying cells, that only certain transient intermediates are TUNEL-reactive, and that mutations in *nuc-1* may block or significantly slow down DNA degradation from proceeding beyond the TUNEL-reactive stage(s). By examining the TUNEL-staining patterns in engulfment-defective mutants, we show that some mutants with previously indistinguishable engulfment-defective phenotypes have distinct TUNEL-staining patterns. These results indicate that some steps in DNA degradation are dependent on engulfment-gene function.
Keywords: Programmed cell death/*C. elegans*/DNA degradation/DNA cleavage/engulfment.

Introduction

Programmed cell death is important for development and homeostasis in metazoans (Ellis *et al.*, 1991b; Steller, 1995; Jacobson *et al.*, 1997). The mechanisms underlying programmed cell death have been the subject of intensive studies of late. One characteristic feature of apoptosis is DNA fragmentation into approximately 180 bp internucleosomal repeats, also referred to as DNA laddering (Wyllie, 1980). Field inversion gel electrophoresis has revealed the cleavage of DNA into 50 kb fragments, presumably at the chromatin interloop domains, which appears to precede the internucleosomal fragmentation (Oberhammer *et al.*, 1993). However, little is known about the identity and regulation of the nucleases in DNA degradation during programmed cell death.

The TUNEL technique (Gavrieli *et al.*, 1992) has been used widely to identify dying cells in many organisms. Terminal deoxynucleotidyl transferase (TdT) is used to label DNA 3'-hydroxyl ends with modified nucleotides detectable by fluorescence (Fluorescein-dUTP) or

immunohistochemistry (digoxigenin-dUTP or biotin-dUTP). TUNEL specifically labels dying cells, which have more DNA ends than viable cells as a consequence of DNA degradation (Wyllie, 1980; Oberhammer *et al.*, 1993). Despite the broad use of TUNEL, several fundamental questions remain to be answered. What is the kinetics of DNA degradation during programmed cell death? Are all intermediates during DNA degradation TUNEL-reactive? Does TUNEL label dying cells in a specific stage of the death process? To answer these questions and to further understand DNA degradation during programmed cell death, we adapted the TUNEL technique for use in *C. elegans*.

Programmed cell death in *C. elegans* is molecularly (Hengartner and Horvitz, 1994; Xue *et al.*, 1996; Zou *et al.*, 1997) and morphologically (Robertson and Thomson, 1982) similar to that of mammals. In *C. elegans*, cells undergoing programmed cell death adopt a refractile, raised button-like morphology, which is readily distinguishable under Nomarski optics. The specific time and place of death is known for each cell genetically programmed to die and is essentially invariant from animal to animal (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). During the development of a hermaphrodite, 131 of 1090 somatic cells generated undergo programmed cell death (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston *et al.*, 1983). Most (113/131) deaths occur during embryonic development, during a short period between 250 and 450 min. after fertilization (Sulston *et al.*, 1983). Combining the TUNEL technique with the detailed knowledge of the cell-death pattern, the kinetics of DNA degradation *in vivo* can be studied in *C. elegans*. In addition, the existence of *C. elegans* mutants defective in different aspects of programmed cell death such as the execution of death (Ellis and Horvitz, 1986; Hengartner *et al.*, 1992) and the engulfment of cell corpses (Hedgecock *et al.*, 1983; Ellis *et al.*, 1991a) allows study of the regulation of DNA degradation in the context of different aspects of cell-death process.

One gene, *nuc-1* (nuclease), which is important for DNA degradation in *C. elegans* has been identified (Sulston, 1976). In *nuc-1* mutants both cell death and engulfment occur, but the pycnotic DNA of dead cells is not degraded and persists as a compact mass of DAPI- or Feulgen-reactive material (Sulston, 1976; Hedgecock *et al.*, 1983). The *nuc-1* gene is also required to digest the DNA of the bacteria on which the animals feed, since

persistent DNA can be detected in the intestinal lumen of *nuc-1* mutants as stained with DAPI (Sulston, 1976).

During programmed cell death, the cell corpses are swiftly engulfed by neighboring cells. At least six genes *ced-1, 2, 5, 6, 7* and *10* have been identified that are important for cell-corpse engulfment (Hedgecock *et al.*, 1983; Ellis *et al.*, 1991a). Mutations in any of these genes block the engulfment of many cell corpses and result in the phenotype of persistent cell corpses. In *ced-1* and *ced-2* mutants Feulgen-reactive material stays in persistent cell corpses (Hedgecock *et al.*, 1983), suggesting that DNA degradation does not proceed or is incomplete in persistent cell corpses. Therefore, the complete digestion of the DNA from dead cells may require the *ced-1* and *ced-2* genes.

Here we describe the TUNEL patterns in wild-type and mutant embryos defective in different aspects of cell death.

Results

The TUNEL technique labels a subset of dying cells in *C. elegans*

We used the TUNEL technique to stain wild-type embryos. We chose embryos at the 1 1/2-fold stage to quantitate the TUNEL-positive nuclei, for two reasons. First, during embryogenesis embryos stay at this easily recognizable stage for less than 20 minutes. Such a small time window allows us to compare the TUNEL-staining patterns from embryo to embryo. Second, by this stage of development 68 cells have diedand been engulfed, based upon the embryonic cell lineage (Sulston *et al.*, 1983). As observed under Nomarski optics, the embryo at this stage has an average of 14 dying cells, which adopt a refractile and raised-button like morphology typical of programmed-cell-death corpses. Therefore, this developmental stage allows study of DNA degradation in both the cells that are actively dying and also those cells that have died earlier and been engulfed. The data presented below are from embryos at the 1 1/2-fold stage.

We found that wild-type embryos at the 1 1/2-fold stage showed on average 1.7 TUNEL-positive nuclei (Table 1; Fig. 1A). Are these TUNEL-positive nuclei those of cells undergoing programmed cell death? Since cell-corpse morphology is not well-preserved in fixed embryos, to address this question, we examined the TUNEL-staining patterns in the embryos of *ced-3*, *ced-4* (Ellis and Horvitz, 1986) and *ced-9*(gf) (Hengartner *et al.*, 1992) mutants in which programmed cell deaths are blocked. We almost never detected

any TUNEL-positive nuclei in these mutants (Table 1), suggesting that these TUNEL-positive nuclei are those of dying cells.

Since in wild-type embryos only an average 1.7 out of 14 dying cells were TUNEL-positive, it is possible that our TUNEL technique is not sensitive enough to detect all ongoing dying cells. However, it is also possible that DNA degradation might be a rapid process and only certain transient intermediates are TUNEL-reactive during the process of cell death. The latter hypothesis is supported by our finding that *nuc-1* embryos have many more TUNEL-positive signals (see below).

TUNEL also labels polar bodies

In wild-type early embryos prior to the 1 1/2-fold stage we occasionally observed the TUNEL staining of polar bodies (Fig. 2A). The *C. elegans* oocyte is arrested at diakinesis of meiotic prophase I. Meiosis is completed after fertilization and two polar bodies are then generated (Hirsh *et al.*, 1976). Our observation suggests that during the degradation of polar-body DNA certain intermediate(s) are TUNEL-reactive. Since the TUNEL staining of polar-body DNA is present in *ced-3, ced-4* and *ced-9*(gf) embryos, *ced-3, ced-4* and *ced-9*(gf) mutations do not prevent all TUNEL-staining, but rather those specifically resulting from programmed cell death.

***nuc-1* embryos have more TUNEL-positive signals than wild-type embryos**

In *nuc-1* mutants programmed cell death and cell-corpse engulfment still occur, but the DNA of dead cells is not completely degraded (Sulston, 1976; Hedgecock *et al.*, 1983). We examined the effect of *nuc-1* mutations on the TUNEL-staining pattern. We observed many more TUNEL-positive signals in *nuc-1* compared with wild-type embryos (Table 2; Fig. 1B). Similar results were obtained using three independently isolated alleles of *nuc-1, e1392, n334* and *n887*, all of which contain undistinguishable number of refractile corpses from wild type when observed using Nomarski microscopy (Table 2).

Are the increased TUNEL-positive signals in *nuc-1* embryos specific to programmed cell death? To address this question, we examined TUNEL patterns of *ced-3; nuc-1(e1392), ced-4; nuc-1(e1392)* and *ced-9*(gf); *nuc-1(e1392)* embryos. We found that these embryos have almost no TUNEL-positive signals (Table 2), consistent with the interpretation that the increased TUNEL-positive signals in *nuc-1* embryos represent pycnotic DNA of cells that died by programmed cell death.

We observed on average 47 TUNEL-positive signals in *nuc-1* embryos, less than the number (68) of cells known to have died by programmed cell death. It is possible that the *nuc-1* alleles tested are not null or that some other endonuclease(s) acts with *nuc-1* in a partially redundant way.

How do mutations in *nuc-1* affect DNA degradation and generate more TUNEL-positive signals than are seen in wild-type embryos? One model is that mutations in *nuc-1* block or significantly slow down DNA degradation from proceeding beyond the TUNEL-reactive stage(s). If so, DNA degradation may involve multiple steps, only some of which are mediated by *nuc-1*.

ced-1 may be required for the generation of TUNEL-positive signals

Since the engulfment genes *ced-1* and *ced-2* may be involved in DNA degradation as suggested by Feulgen staining in persistent cell corpses of *ced-1* and *ced-2* mutants (Hedgecock *et al.*, 1983), we examined the TUNEL-staining patterns in the engulfment mutants *ced-1, 2, 5, 6, 7* and *10*.

We found that *ced-1* and *ced-1; nuc-1(e1392)* embryos have very few TUNEL-positive nuclei (Table 3). Similar results have been obtained using two independently isolated *ced-1* alleles, *e1735* and *n1995*. These results suggest that *ced-1* activity may be essential for the generation of TUNEL-reactive DNA ends during programmed cell death.

Mutations in _ced-7_ may partially block the generation of TUNEL-positive signals

We found that *ced-7* embryos have fewer TUNEL-positive signals than wild-type embryos and *ced-7; nuc-1(e1392)* embryos have less than half the TUNEL-positive signals of *nuc-1* embryos (Table 4). Similar results were obtained using three different *ced-7* alleles, *n1996, n1892* and *n2094,* suggesting that the reduction of TUNEL-positive signals in *ced-7* mutants is specifically caused by the mutations in the *ced-7* locus. The nature of molecular lesions and western analysis using anti-CED-7 antibodies suggest that the *n2094* and *n1996* alleles are likely to be null (Y.-C. Wu and H.R.H., unpublished results). Therefore, *ced-7* may act with other gene(s) in a partially redundant way to regulate the generation of TUNEL-reactive DNA ends during programmed cell death.

The *ced-7* gene encodes a protein similar to ABC (ATP-binding cassette) transporters (Y.-C. Wu and H.R.H., unpublished results). Since the CED-7 protein appears to be localized to the plasma membrane by

immunostaining with anti-CED-7 antibodies (see chapter 3), CED-7 is unlikely to be directly involved in DNA degradation in the nucleus. The identification and characterization of substrates transported by CED-7 should help to understand how *ced-7* functions in both engulfment and DNA degradation.

Mutations in *ced-2, 5, 6* and *10* do not alter numbers of TUNEL-positive signals

Unlike *ced-1* and *ced-7* mutants, we found that *ced-2, 5, 6* and *10* mutants have indistinguishable numbers of TUNEL-positive nuclei from those in wild type (Table 5). Furthermore, *ced-2; nuc-1(e1392), ced-5; nuc-1(e1392), ced-6; nuc-1(e1392)* and *ced-10; nuc-1(e1392)* embryos have indistinguishable numbers of TUNEL-positive nuclei from those in *nuc-1* embryos (Table 5). These results suggest that the engulfment genes *ced-2, 5, 6* and *10* do not affect the DNA-degradation process by which TUNEL-reactive DNA ends are generated and degraded. Therefore, the endonuclease(s) generating TUNEL-reactive DNA ends and the nuclease(s) regulated by *nuc-1* to destroy TUNEL-reactive DNA ends are probably expressed and act in dying cells rather than being provided by engulfing cells.

In *ced-2; nuc-1(e1392), ced-5; nuc-1(e1392), ced-6; nuc-1(e1392)* and *ced-10; nuc-1(e1392)* embryos, we frequently observed the TUNEL-positive signals in the unengulfed cell corpses which detached from embryos and are shed into egg fluid (Fig. 3). This observation further suggests that the generation of TUNEL-reactive DNA ends may occur inside dying cells independent of engulfing cells.

DNA degradation may require the participation of engulfing cells during programmed cell death

Although our TUNEL data suggests that in *ced-2* mutants the DNA-degradation process by which TUNEL-reactive DNA ends are generated and degraded may proceed normally, the Feulgen staining in persistent cell corpses of *ced-2* mutants indicates that DNA degradation is incomplete (Hedgecock *et al.*, 1993). Therefore, *ced-2* may be involved in DNA degradation where it acts downstream of *nuc-1*. To examine if incomplete DNA degradation also occurs in unengulfed cell corpses of *ced-5, 6, 7* and *10* mutants, we stained these mutants with the DNA-binding dye SYTO 11. We detected SYTO 11-reactive material in all persistent cell corpses of *ced- 5, 6 , 7* and *10* mutants (n=30 for each mutant)

(data not shown). Therefore, it is possible that some steps of the DNA degradation downstream of *nuc-1* may occur inside engulfing cells.

Discussion

We have adapted the TUNEL technique for use in *C. elegans* to specifically label dying cells. The majority (47/68) of pycnotic DNA from dead cells in *nuc-1* embryos are TUNEL-positive, arguing that the TUNEL technique in *C. elegans* is sensitive enough to label the majority of TUNEL-reactive DNA ends. However, in wild-type embryos only a small subset of dying cells are TUNEL-positive, suggesting that DNA degradation is a rapid process and that only certain transient intermediates are TUNEL-reactive. Therefore, TUNEL technique may normally label dying cells during a brief, highly specific stage of programmed cell death.

We showed that DNA degradation during programmed cell death may involve at least three steps (Fig. 4). The first step is mediated by the genes *ced-1* and *ced-7* to generate TUNEL-reactive DNA ends. Mutations in either gene reduce the TUNEL-positive signals. The second step involves the *nuc-1* gene, which may mediate the conversion of TUNEL-reactive DNA ends into TUNEL-unreactive ones. The nucleases involved in the first two steps may act in dying cells rather than in engulfing cells, and *ced-1*, *ced-7* and *nuc-1* therefore may act in dying cells to regulate the nuclease activities. The third step involves the further degradation of TUNEL-unreactive DNA fragments, which may occur in engulfing cells.

How are TUNEL-reactive DNA ends generated during programmed cell death? The time-course studies of various apoptotic epithelial cells during the death process using gel electrophoresis suggest that DNA degradation involves multiple steps (Oberhammer *et al.*, 1993). The chromosomal DNA of some apoptotic cells is cleaved at the chromatin loop domains, generating 50 kb fragments (Oberhammer *et al.*, 1993). The subsequent cleavage of DNA at the internucleosomal linker region produces DNA fragments of approximately 180 bp repeats (Wyllie, 1980; Oberhammer *et al.*, 1993). Two recent findings suggest that the DNA fragments generated by DNA laddering may be TUNEL-reactive. First, the DNA fragments generated by DNA laddering contain 3'-hydroxyl ends in apoptotic lymphocytes (Alnemri and Litwack, 1990). Second, the kinetics of TUNEL-positive signals appear to correlate well with that of the DNA laddering

in dexamethasone-treated apoptotic thymocytes in culture (Gavrieli *et al.*, 1992).

What molecules mediate DNA laddering? The cytosolic heterodimeric protein DFF (DNA fragmentation factor) triggers DNA laddering *in vitro* as DFF is activated by the proteolytic cleavage by apoptotic caspase-3 (Liu *et al.*, 1997). Purified DFF appears to have no inherent DNAse activity, suggesting that it may instead regulate DNAse(s) to mediate DNA laddering (Liu *et al.*, 1997). The endonucleases deoxyribonuclease I (DNAse I) (Peitsch *et al.*, 1993; Peitsch *et al.*, 1994), NUC18 (Montague *et al.*, 1994), deoxyribonuclease II (DNAse II) (Barry and Eastman, 1992; Barry and Eastman, 1993), inducible-lymphocyte Ca^{2+}/Mg^{2+}-dependent-endonuclease (ILCME) (Khodarev and Ashwell, 1996) and cyclophilins (Montague *et al.*, 1997) have been implicated in DNA degradation during programmed cell death. DNAse I, ILCME and cyclophilins generate 3'-hydroxyl ends, consistent with the hypothesis that they may generate TUNEL-reactive ends during programmed cell death. However, DNAse II produces 3'-phosphate and 5'-hydroxyl ends, neither of which are substrates of TdT in the TUNEL reaction. Eleven cyclophilin genes have been identified in *C. elegans* (Page *et al.*, 1996). Future reverse genetics and biochemical assays may help to determine if any of them play a role in DNA degradation during programmed cell death.

How does *nuc-1* mediate DNA degradation? Our data suggest that *nuc-1* may act to degrade TUNEL-reactive DNA ends. As mentioned earlier, TdT synthesize a polydeoxynucleotide polymer at 3'-hydroxyl ends. Therefore, *nuc-1* might destroy or mask these TUNEL-reactive 3'-hydroxyl ends. It has been suggested that an endonuclease activity, detected in the wild-type but undetectable from *nuc-1* protein extracts, may be regulated by *nuc-1* (Hevelone and Hartman, 1988). This endonuclease activity resembles that of deoxyribonuclease II (DNAse II) in that they both are independent of Ca^{2+} and Mg^{2+} and have a characteristic acidic pH optimum (Hevelone and Hartman, 1988; Barry and Eastman, 1992). In addition, the degradation of DNA by DNAse II generates 5'-hydroxyl and 3'-phosphate ends, neither of which are substrates of TdT in the TUNEL reaction, consistent with the hypothesis that *nuc-1* activity does not generate TUNEL-reactive DNA ends. It is possible that *nuc-1* may regulate at least two activities: one to destroy or mask TUNEL-reactive DNA ends and the other similar to DNAse II endonuclease.

Materials and methods

Strains

All strains were grown on Petri dishes seeded with bacteria OP50 at 20°C. All mutations were generated in a Bristol N2 background, which we used as the standard wild-type strain. The following mutations, which were not mentioned above, were used: LGIII *ced-4(n1162), ced-9(n1950)* and LGIV *ced-3(n717).*

TUNEL method

Animals were washed off 1 to 3 100X50 mm plates and hypochlorite-treated to obtain embryos. Embryos were fixed using a modified protocol derived from Finney and Ruvkun (1990). In brief, 1 ml of fixation solution containing 80 mM KCl, 20 mM NaCl, 1.3 mM EGTA, 3.2 mM spermine, 7.5 mM sodium Hepes pH 6.5, 25% methanol, 2% paraformaldehyde, 0.4% glutaraldehyde was added to hypochlorite-treated embryos, which were then immediately frozen in liquid nitrogen. The frozen embryos were thawed in water bath at room temperature for 2 minutes and rocked at room temperature for 25 minutes. Fixed embryos were then washed once in 1 ml Tris-Triton buffer (1% Triton-X100 and 100 mM Tris, pH7.4) and three times in 1 ml PBST (1X PBS containing 0.5% Triton-X100). Approximately 3 ml of packed embryos were preincubated with 25 ml of TdT reaction buffer (200mM sodium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml bovine serum albumin, 0.1% Triton X100, 1.5 mM cobalt chloride, pH 6.6) for 5 minutes at room temperature. The TdT reaction buffer was then replaced with the TdT reaction buffer containing 0.2 unit of TdT (Boehringer Mannheim), 6.6 nM dUTP (Boehringer Mannheim) and 3.3 nM fluorescein-11-dUTP (Boehringer Mannheim) and incubated for 2 hrs. at 37°C. After incubation, embryos were washed in PBST as before. Embryos were then mounted in VECTORSHIELD mounting medium (Vector Laboratories) and visualized using either a Zeiss microscope equipped for fluorescence microscopy or a Bio-Rad MRC-500 confocal microscope.

Cell-corpse count

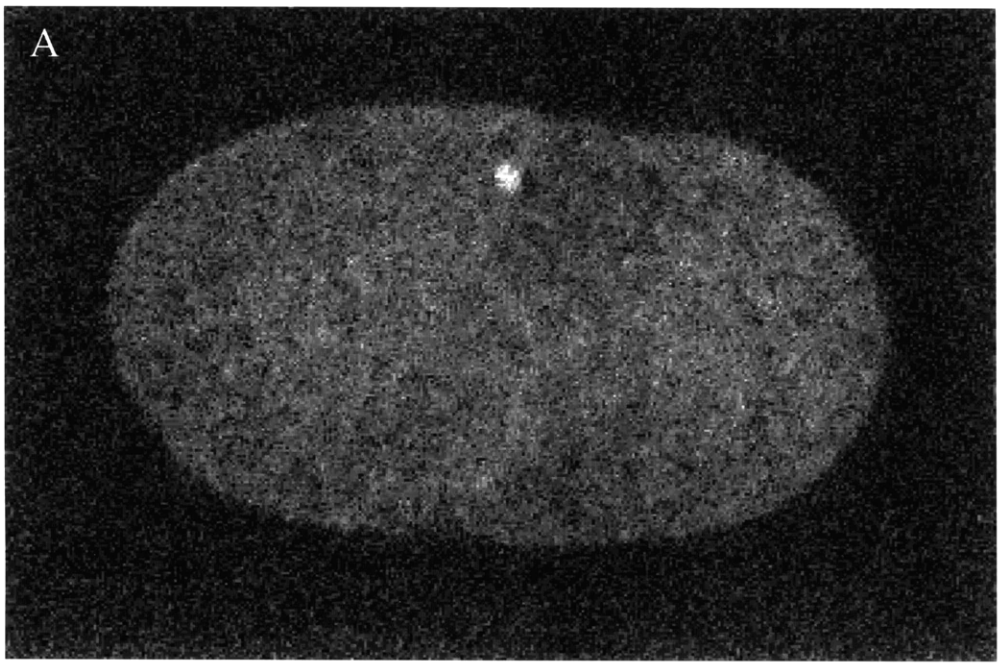
The number of cell corpses in living embryos at the 1 1/2-fold stage were counted using Nomarski optics, as previously described (Ellis *et al.*, 1991a).

SYTO 11 staining

Worms of each genotype were grown on a 100x50 mm plate. Worms were washed off plates with M9 solution, collected in a 1.5 ml eppendorf tube, and washed once in M9. The washed worms were then incubated with 1 ml 10 nM SYTO 11 (Molecular Probe) in M9 and were rocked at room temperature for 1.5

hrs. The stained worms were washed once in M9 and spot onto a plate for recovery for more than 30 mins. Worms were mounted on 4% agar pads with 20 mM NaZ_3 and visualized using a Zeiss microscope equipped for fluorescence microscopy and using Nomarski optics to score cell corpses.

Fig. 1. A wild-type and a *nuc-1* embryo show different TUNEL-staining patterns. A wild-type embryo (**A**) and a *nuc-1* embryo (**B**) at the 1 1/2-fold stage stained with TUNEL were photographed using the Bio-Rad MRC-500 confocal microscope. Some of TUNEL signals in *nuc-1* embryos are smaller than those seen in wild type and are likely to be those of pycnotic DNA from dead cells. Each picture is a projection of 8 serial confocal images.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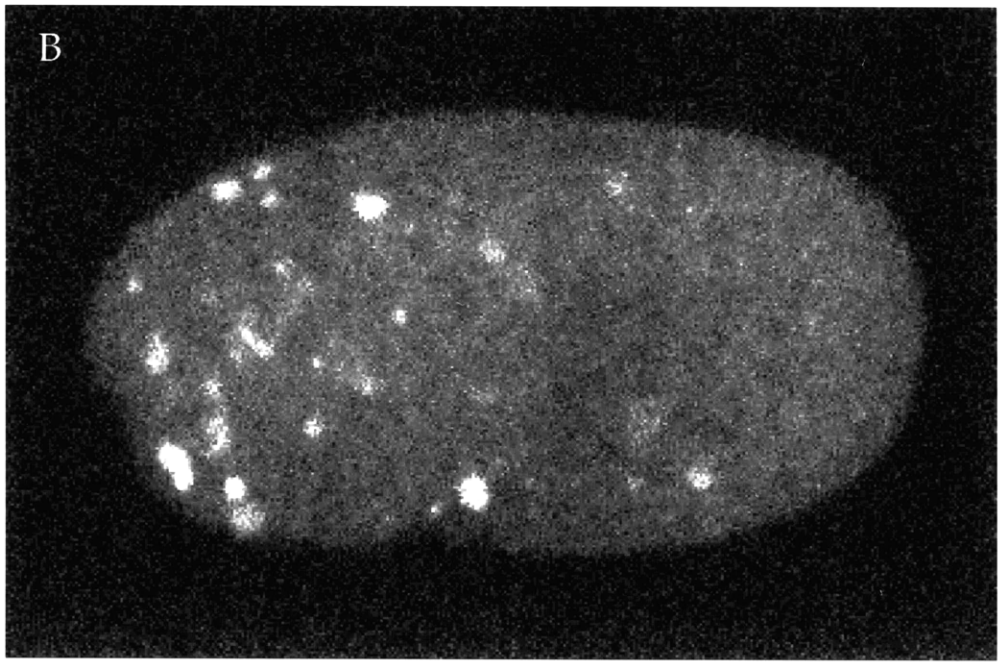
B

Fig. 2. An early wild-type embryo showing TUNEL staining of a polar body. A wild-type embryo stained with TUNEL(**A**) or DAPI (**B**). The polar body is indicated by arrows.

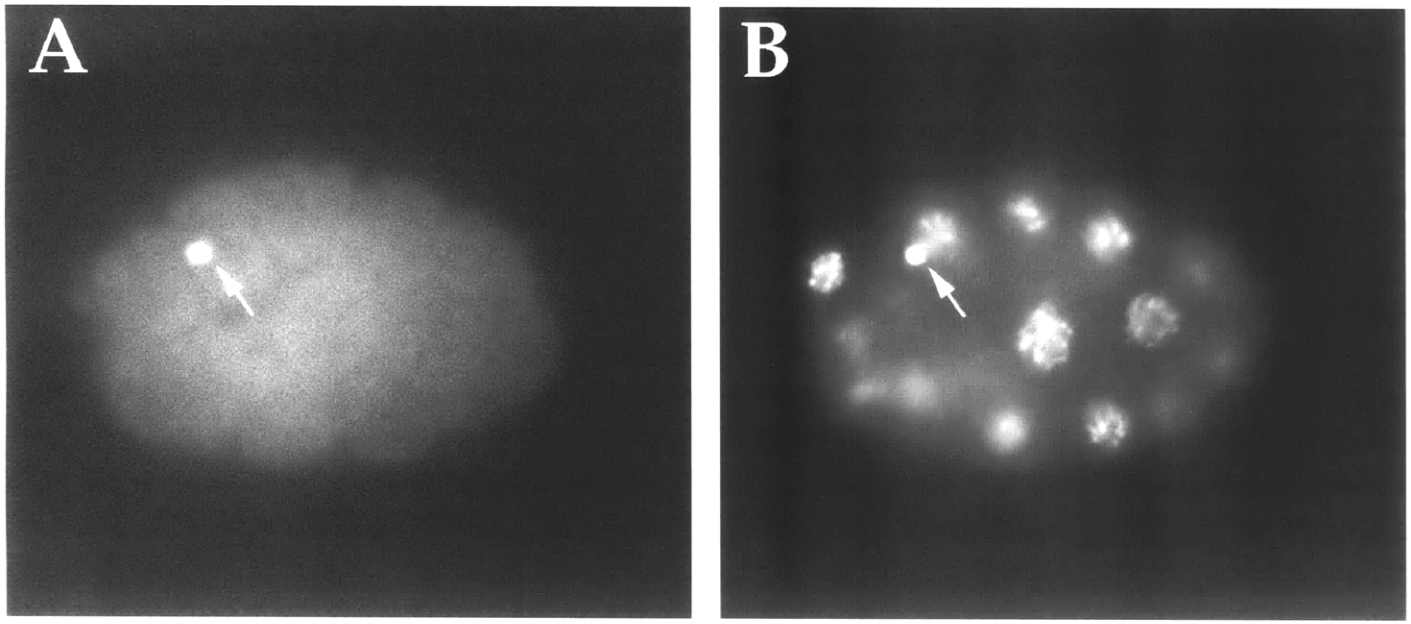
A
B

Fig. 3. Unengulfed cell corpses in engulfment-defective mutants are TUNEL-positive.
(**A**) A *ced-6(n1813); nuc-1(e1392)* embryo stained with TUNEL.
(**B**) The same embryo observed using Nomarski microscopy. A TUNEL-positive unengulfed cell corpse excluded from the embryo is indicated by arrows.

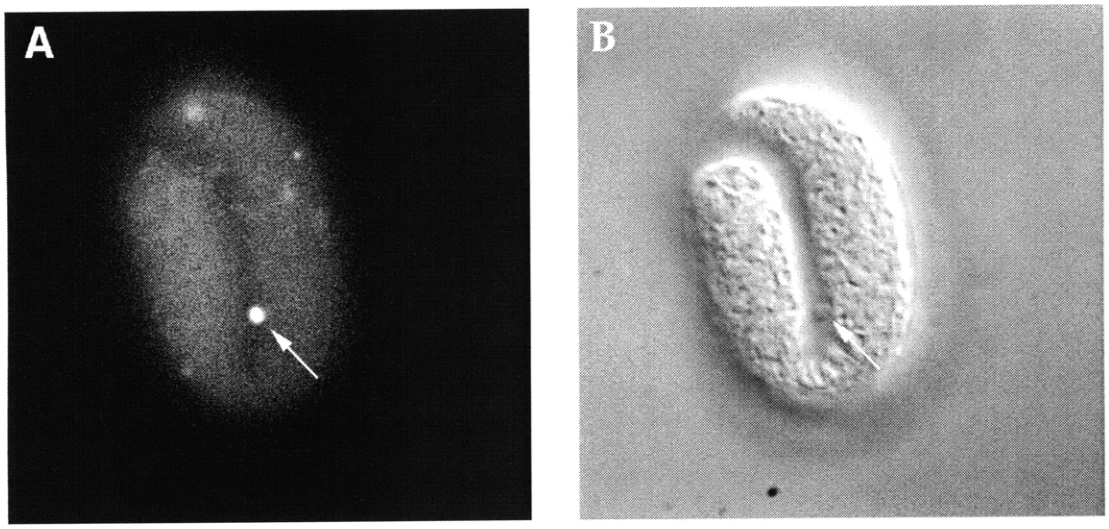
A
B

Fig. 4. Model for DNA degradation during programmed cell death in *C. elegans*. Each arrow may represent a number of parallel or sequential nuclease activities which are regulated by *ced-1, 2, 5, 6, 7, 10* or *nuc-1* as indicated.

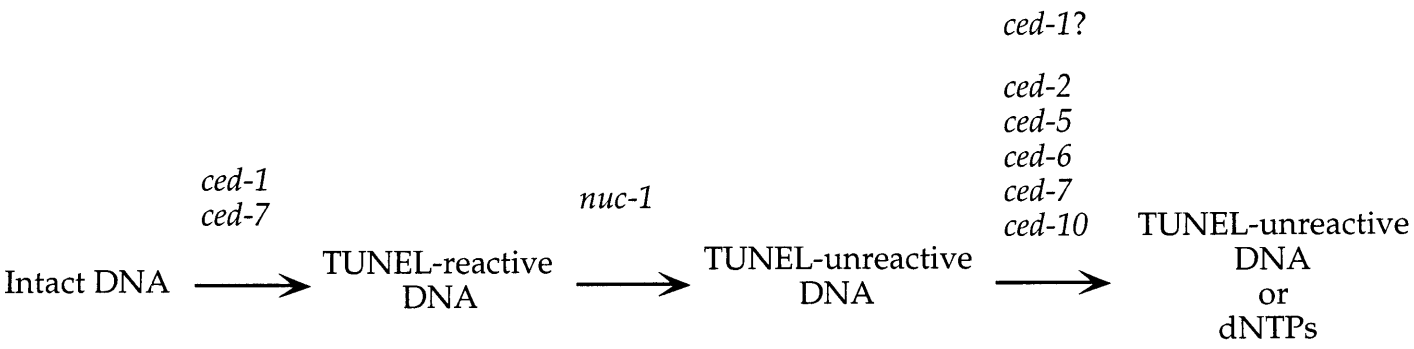
ced-1?
ced-2
ced-5
ced-6
ced-7
ced-10
ced-1
ced-7
nuc-1
Intact DNA
TUNEL-reactive DNA
TUNEL-unreactive DNA
TUNEL-unreactive DNA or dNTPs

Table 1 TUNEL specifically labels dying cells

Strain	No. of TUNEL-positive cells*	Range of TUNEL-positive cells	No. of cell corpses#
wild-type	1.7 ± 1.3	0-4	14 ± 1
ced-3	0.1 ± 0.2	0-1	0.0 ± 0.0
ced-4	0.0 ± 0.0	0-1	0.0 ± 0.2
ced-9(gf)	0.1 ± 0.4	0-1	0.1 ± 0.3

* TUNEL-positive nuclei in at least 60 embryos of each genotype were counted.

Cell corpses in 15 wild-type embryos and more than 50 embryos of each mutant genotype were counted using Nomarski optics.

All data were scored from embryos at 1 1/2-fold stage. The data shown are means ± s.e.m.

Table 2 TUNEL labels persistent DNA in *nuc-1* animals

Strain	No. of TUNEL-positive cells (n=45)	Range of TUNEL-positive cells	No. of cell corpses (n=15)
nuc-1(e1392)	47.8 ± 4.8	38-64	14 ± 2
nuc-1(n334)	45.3 ± 6.2	36-60	14 ± 2
nuc-1(n887)	47.5 ± 5.3	35-59	14 ± 1
ced-3; nuc-1(e1392)	0.4 ± 0.7	1-2	0.0 ± 0.0
ced-4; nuc-1(e1392)	0.2 ± 0.6	1-2	0.1 ± 0.2
ced-9(gf); *nuc-1(e1392)*	4.2 ± 1.8	1-9	0.1 ± 0.4

All data were scored from embryos at 1 1/2-fold stage. The data shown are means ± s.e.m.

Table 3 Mutations in *ced-1* gene significantly reduce TUNEL-positive signals

Strain	No. of TUNEL-positive cells (n=45)	Range of TUNEL-positive cells	No. of cell corpses (n=15)
ced-1(e1735)	0.5 ± 0.7	0-3	24 ± 3
ced-1(n1995)	0.3 ± 0.2	0-2	25 ± 4
ced-1(e1735); nuc-1(e1392)	1.0 ± 1.0	0-3	26 ± 3
ced-1(n1995); nuc-1(e1392)	1.2 ± 1.0	0-5	27 ± 5

All data were scored from embryos at 1 1/2-fold stage. The data shown are means ± s.e.m.

Table 4 *ced-7* mutants have fewer TUNEL-positive signals

Strain	No. of TUNEL-positive cells (n=45)	Range of TUNEL-positive cells	No. of cell corpses (n=15)
ced-7(n2094)	0.7 ± 0.8	0-3	34 ± 3
ced-7(n1892)	0.5 ± 0.4	0-2	35 ± 5
ced-7(n2094); nuc-1(e1392)	21.1 ± 1.3	15-28	38 ± 3
ced-7(n1892); nuc-1(e1392)	22.3 ± 3.2	14-29	37 ± 5
ced-7(n1996); nuc-1(e1392)	19.0 ± 1.7	16-26	38 ± 4

All data were scored from embryos at 1 1/2-fold stage. The data shown are means ± s.e.m.

Table 5 Mutations in *ced-2, 5, 6* and *10* do not alter TUNEL-staining

Strain	No. of TUNEL-positive cells (n=45)	Range of TUNEL-positive cells	No. of cell corpses (n=15)
ced-2(e1752)	1.5 ± 1.1	0-3	21 ± 3
ced-2(n1994)	1.7 ± 1.4	0-3	20 ± 3
ced-5(n1812)	1.5 ± 1.0	0-4	39 ± 5
ced-5(n2691)	1.4 ± 1.2	0-3	34 ± 4
ced-6(n2095)	1.4 ± 0.9	0-3	29 ± 4
ced-6(n1813)	1.6 ± 1.4	0-3	33 ± 5
ced-10(n1993)	1.4 ± 1.3	0-3	31 ± 4
ced-2(e1752); nuc-1(e1392)	44.6 ± 4.6	37-51	23 ± 3
ced-2(n1994); nuc-1(e1392)	47.6 ± 4.9	41-56	21 ± 3
ced-5(n1812); nuc-1(e1392)	45.1 ± 5.8	39-59	38 ± 5
ced-5(n2691); nuc-1(e1392)	44.6 ± 4.6	35-56	35 ± 4
ced-6(n2095); nuc-1(e1392)	46.2 ± 4.5	33-54	30 ± 5
ced-6(n1813); nuc-1(e1392)	43.6 ± 4.6	35-52	32 ± 5
ced-10(n1993); nuc-1(e1392)	42.6 ± 4.3	37-51	33 ± 4

All data were scored from embryos at 1 1/2-fold stage. The data shown are means ± s.e.m.

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